Thesis for the Degree of Doctor of Engineering

Characterization of biochemical and biofunctional properties of γ-PGA produced from halotolerant marine *Bacillus* sp.

By

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August 26, 2016

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해양 유래 내염성 *Bacillus*가 생산하는 γ-PGA의 생화학적 효과 및 기능성에 관한 연구

Advisor: Prof. In-Soo Kong

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A thesis submitted in partial fulfillment of the requirements
for the degree of Doctor of Engineering
In Department of Biotechnology, The Graduate School,
Pukyong National University

August 2016

이종민의 공학박사 학위논문을 인준함.

2016년 8월 26일

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Characterization of biochemical and biofunctional properties of γ-PGA produced from halotolerant marine *Bacillus* sp.

A dissertation

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August 26, 2016

BRIEF CONTENTS

Characterization of biochemical and biofunctional properties of γ-PGA produced from halotolerant marine *Bacillus* sp.

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Abstract

The marine-derived spore former halotolerant *Bacillus* sp. SJ-10 isolated from *jeotgal*, a traditional Korean salt-fermented seafood, was evaluated *in vitro* as human and animals probiotics, in addition to demonstrate the antioxidant mechanism and protection effects against hydroxyl radical induced oxidative damage of poly-γ-glutamic acid (γ-PGA) produced by it.

The *Bacillus* sp. SJ-10 was demonstrated on safety against cytotoxicity, hemolysis and susceptibility on all tested antibiotics. It met the prerequisite as probiotics by showing that the spore counts are remained almost constant with acid and bile tolerance during incubation in simulated gastrointestinal condition, and exhibited significant adhesion to caco-2 cell by its sureface hydrophobicity and autoaggregation capacity. This strain showed beneficial properties as probiotics; broad spectrum antimicrobial activity to both gram-negative and gram-positive in particular marine-borne pathogens, hydroxyl radical scavenging antioxidant activity, production of functional enzymes such as β-galactosidase, phytase, bile salt hydrolase and catalase, and the availability of selective growth through fermentation of barley β-glucan as well as inulin and FOS.

Moreover, extracellular viscous biopolymer produced by SJ-10 was purified and characterized. By physicochemical analysis using FT-IR, NMR, thermal and amino acid analysis, the

biopolymer was indicated that γ-PGA is a homopolymer mostly composed of L-glutamic acid with sodium salt form with constant molecular weight of ~400kDa in 8% NaCl fermentation condition. The sodium poly-γ-glutamic acid with molecular weight of 400 kDa was found to have strong hydroxyl radical averting capacity by chelating metal ions and scavenging hydrogen peroxide. The γ-PGA showed significantly protective effect to DNA, protein and Caco-2 cell on oxidative damage by hydroxyl radical.

According to the obtained results through in vitro assessments, the *Bacillus* sp. SJ-10 demonstrated that it is possible to successfully use these probiotics to human and animals. In particular, it is available as a promising alternative to antibiotics and health enhancing agents for use as a feed additive and food supplements in aquaculture and seafood fermentation, respectively.

Chapter 1

In vitro evaluation of the marine-derived spore-former as promising probiotics candidate

Abstract

The marine-derived spore-former *Bacillus* strain was evaluated in vitro prior to in vivo test for application as human and animal probiotics. *Bacillus* sp. SJ-10 was demonstrated on safety against cytotoxicity, hemolysis and susceptibility on all tested antibiotics. It met the prerequisite as probiotics by showing that the spore counts are remained almost constant with acid and bile tolerance during incubation in simulated gastrointestinal condition, and exhibited significant adhesion to caco-2 cell by its sureface hydrophobicity and autoaggregation capacity. Moreover, this strain showed beneficial properties as probiotics; broad spectrum antimicrobial activity in particular marine-borne pathogens, hydroxyl radical scavenging antioxidant activity, production of functional enzymes such as β-galactosidase, phytase, bile salt hydrolase and catalase, and the availability of selective growth through fermentation of β-glucan as well as inulin and FOS. According to the obtained results through in vitro assessments, the strain SJ-10 was demonstrated that it is possible to successfully use as probiotics to human and animals. In particular, it is available as a promising alternative to antibiotics and health enhancing agents for use as a feed additive and food supplements in aquaculture and seafood fermentation, respectively.

Keywords; Bacillus probiotics, Bacterial adhesion, Acid resistance, Antimicrobial activity, Antioxidant

1. **Introduction**

In order to obtain health benefits from valuable organism, the growing health awareness in the consumption of microorganisms as probiotics has been increased world-widely. According to the WHO/FAO, probiotics are "Live microorganisms which when administered in adequate amounts confer a health benefit on the host", that is, which are dietary supplements and live microorganisms containing potentially beneficial microorganisms [1, 2].

Lactobacillus and *Bifidobacterium* strains are the most extensively used probiotics bacteria, and these Lactic acid bacteria (LAB) have been particularly used in biomedical and food industry until now because they are able to convert sugars and other carbohydrates into lactic acid. They must promote that beneficially affect the host by improving the properties of the indigenous flora and inhibit enteropathogens, without causing any undesirable effects. Additionally, probiotics must be metabolically stable and well active during manufacturing, storage and administration conditions so they can be successful in the passage through upper digestive tract and then in adhering and colonizing the intestine system. However, the growth activity of LAB is affected by fermentation conditions such as temperature, pH, in fermented products during storage and stability of the dried and frozen form [3]. Recently, useful approaches that enhance the resistance of these sensitive microorganisms against adverse conditions have been proposed including use of oxygen impermeable containers, stress adaptation, incorporation of micronutrients such as peptides and amino acids and microencapsulation [4]. Nevertheless, because these techniques results in additional costs, the necessity of appropriate probiotics selection for economic and effective alternative resistant strain against acid and bile, heat, lyophilization and dehydration has increased.

Meanwhile, less known genus than lactic acid bacteria as probiotics are certain spore-formers, mainly the genus *Bacillus* species, which are being used to both human and animals. Among a variety of species in probiotics, those belonging to the genus *Bacillus* have the advantage since their ability to sporulate and germinate. They survive at harsh conditions during desiccation by

methods that involve moderate heating, such as spray dryers, avoiding the use of lyophilization or other expensive technologies as well as passage of the gastrointestinal tract in the presence of bile salts with acidic pH [5]. This property also makes possible the administration of spores mixed with powdered vehicles instead of gels or liquids used with non-sporulated bacteria. These products in their spore form have been shown to elicit potent immune responses and prevent gastrointestinal disorders by suppressing infection with pathogen [6]. Furthermore, there is now strong evidence for their use in treating and preventing some human diseases as well as animals, and the diversity of species used and their applications are astonishing. [7-9].

Salted-fermented seafood (*jeotgal*), fermented vegetables (*kimchi*) and soybean paste (*doenjang*) are the representative traditional foods served as side dishes in Korea. These traditional fermented foods have been a major source of various nutrients in the Korean diet for thousands of years. Among them, *joetgal* is prepared through adding 20-30% (w/w) salt and blending of various kinds of seafood (squid, shrimp, shellfish, oyster, fish, fish eggs or intestines) and becomes palatable through subsequent preservation and fermentation. Possible sources of the fermentation inocula of *jeotgal* include organisms that occur naturally on or in marine animals, organisms associated with the animals' environment (e.g., seawater or marine mud), terrestrial organisms not normally associated with the marine environment, and organisms associated with the natural microflora of the marine salt used in preparing the seafood for fermentation. Hence, *jeotgal* is a large source of microorganisms, as indicated by the isolation of 19 novel species in this product since 2000 [10]. However, the exact role of each microorganism in the health benefit, in particular squid *jeotgal*, has not yet been established, although the diversity of microorganisms in various *jeotgal* has been demonstrated. Recently, there are effort to use as probiotics of *Bacillus* species isolated from squid *jeotgal* since its significantly excellent functionality which inhibition of food pathogens and excellent producer of S-adenosyl-L-methionine known as a nutritional supplement to improve brain functions of the human as well as a essential substance for the synthesis, activation, and

metabolism of hormones, neurotransmitters, nucleic acids, phospholipids, cell membranes of animals [11, 12].

Therefore, the aim of this study was to assess the potential usefulness of *Bacillus* sp. SJ-10 isolated squid *jeotgal* (also called *Ojingeo-jeotgal* in Korea) as probiotics in vitro evaluation, and to point to the direction for in vivo future research and application.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Strains of *Bacillus* spp., laboratory identified as SJ-10 (JCM 15709, KCCM 90078), was originally isolated from squid *jeotgal* [13]. The strain was routinely cultured in halophiles medium (HM), which contains 0.1% glucose, 0.5% yeast extract, 0.5% proteose peptone and 5% NaCl, at 37℃ overnight. For the reference strains, the four probiotics strains *Lactobacillus casei* KCTC 3109, *L. fermentum* KCTC 13097, *L. plantarum* ATCC 14917 and *L. rhamnosus* ATCC 7469 were used and cultured in MRS medium at 37℃ in anaerobic atmosphere with 5% CO_2. *Edwardsiella ictaluri* ATCC 33202, *E. tarda* ATCC 12267, *Vibrio anguillarum* serotype O1, *V. alginolyticus* KCTC 2472, *V. cholera, V. parahaemolyticus* KCTC 2471, *V. vulnificus* KCTC 2985, *Salmonella typhi, Shigella flexneri, S. sonnei, Escherichia coli* O157, *Aeromonas hydrophila* KCTC 2358, *A. salmonicida, Listeria monocytogenes* KCTC 3071, *L. monocytogenes* KCTC 13064, *Bacillus cereus* KCTC 3624, *B. cereus* KCTC 1012, *Streptococcus iniae* KCTC 3657, *S. mutans* KCTC 3065, *Staphylococcus aureus* subsp. *aureus* and *Lactococcus garvieae* KCTC 3772 were used as the indicator strains. Source of *Salmonella typhi, Shigella flexneri, S. sonnei* and *A. salmonicida* are laboratory collection, and these strains have been identified using 16s rRNA sequencing. Pathogens were grown on BHI medium for 24 h at 37℃ exception some *Vibrio* strains (25℃).

2.2. Caco-2 cell culture

Heterogeneous human epithelial colorectal adenocarcinoma (Caco-2) cells (KCLB 30037) were obtained from the Korean Cell Line Bank (KCLB) and used for experiments between passages 10 and 20. The cells were grown at 37℃ in an atmosphere of 95% air and 5% CO_2 in minimal essential medium (MEM, Sigma M0643) supplemented with 25 mM HEPES, 10% heat-inactivated fetal bovine serum (FBS, Gibco 16000), 1% non-essential amino acids and 1% penicillin/streptomycin (Gibco, Grand Island, NY). Confluent monolayers were subcultured every

4 - 5 days by treatment with 0.25% trypisn and 0.2% EDTA in phosphate buffer saline.

2.3. Preparation of spores

The sporulation was performed by batch fermentation. Briefly, overnight cultured SJ-10 in HM was inoculated with 1% (v/v) of seed inoculum into 100 mL of sporulation media (8 g/L Bacto nutrient broth, 1 g/L KCl, 0.12 g/L $MgSO_4 \cdot 7H_2O$, 0.16 g/L $Ca(NO_3)_2$, 1.2 mg/L $MnCl_2$, 0.27 mg/L $FeSO_4$, pH 7.2) and incubated on a rotary shaker for 7 days at 37℃ with 250 rpm. After incubation, spores were harvested by centrifugation and washed 5 times in ice-cold water. To kill any residual vegetative cells, sporangial cells or germinated spores, the suspensions of spores were shaken for 5min by ultrasonic wave at the setting duty-cycle 40%, output control 4 (Microtip, Branson, Sonifier 250). Next, Spores were washed repeatedly with distilled water (twice), concentrated by centrifugation, and heat treated at 65℃ (45 min) to kill any residual vegetative cells, sporangial cells or germinated spores. Each spore suspension was titrated immediately to determine the number of colony forming unit (CFU) per milliliter before aliquots were frozen at -80℃ until use.

2.4. Safety assessment

2.4.1. Cytotoxicity assay

A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was conducted to analyze the cytotoxicity of SJ-10 using the Cell Titer 96 ® Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega). Caco-2 cells (~5×10^4) were seeded in 96-well cell plates and cultured with maintenance medium at 37℃ in 5% CO_2 atmosphere. After 24 h, the culture medium was changed with fresh 100 μL medium supplied with same volume of each cultured medium of *Bacillus* sp. SJ-10, *L. plantarum* ATCC 14917 (as a probiotics control), *B. cereus* KCTC 1012 and *S. typhi* (as a pathogenic controls). These bacteria cultured media were added into the wells and incubated further for 24 h. The cultured media were obtained by centrifugation and filtration through a 0.2 μm syringe filter of 18 h bacterial cultures

in Luria-Bertani (LB). The cells were stimulated for 24 h. Then, 20 μL of MTS solution (5 mg/mL) was added to each well and cultured for 1 h. The absorbance was evaluated at 570 nm using a multi detection microplate reader.

2.4.2. Hemolytic tests

Haemolysis was determined on brain heart infusion agar supplemented with 10% sheep blood after incubation at 37℃ for 24 h.

2.4.3. Antibiotic susceptibility

The susceptibility of the SJ-10 to streptomycin, kanamycin, cabenicillin, chloramphenicol, tetracycline, penicillin and ampicillin were determined in LB agar plates with paper disc.

2.5. Acid and bile salt tolerance during simulated gastrointestinal condition

The simulated gastrointestinal condition was adapted from previous reported with certain modifications [14, 15]. The spores (~10^8 CFU/mL) were transferred to clean amber bottles and mixed with saline to create a final volume of 19 mL. The samples were acidified to pH 2 with 1 mL of a porcine pepsin preparation (0.04 g pepsin in 1 mL 0.1 mol/L HCl), and incubated at 37℃ in a shaking water bath at 95 rpm for 3 h. After during the simulated gastric condition, the pH was increased to 5.3 with 0.9 mol/L sodium bicarbonate followed by the addition of 200 μL of bile salts glycodeoxycholate (0.15 g in 1 mL saline), taurodeoxycholate (0.1 g in 1 mL saline), taurocholate (0.15 g in 1 mL saline), and 100 μL of pancreatin (0.04 g in 500 μL saline). The pH of each sample was increased to 8 with 10N NaOH. Samples were incubated in a shaking water bath (95 rpm) at 37℃ for 8 h to complete the intestinal phase of the in vitro digestion process. After the intestinal phase, appropriate dilutions were plated directly onto MRS agar plates. These plates were incubated for 24 h at 37℃ to determine $\log_{10}$CFU/mL.

2.6. Bacterial adhesion

2.6.1. Cell surface hydrophobicity

The hydrophobicity and autoaggregation assay were carried out according to Kos et al., 2003,

with some modifications [16]. Briefly, the value of hydrophobicity of the SJ-10 was determined based on adhesion of cells to organic solvents. The cultures were grown in 10 mL of MRS broth, centrifuged at 6000 rpm for 5 min for cell separation. The pellet was washed and re-suspended in 10 mL of Ringer's solution, and then absorbance of this aqueous phase at 600 nm as Ao was measured. Cell suspension was mixed with equal volume of solvent and mixed thoroughly by vortexing for 2 min. Hexadecane and xylene, chloroform and ethyl acetate were used as an apolar solvent, a monopolar and acidic solvent, a monopolar and basic solvent, respectively. The two phases were allowed to separate for 30 min at room temperature, and absorbance at 600 nm of non aqueous was recorded as A1. The hydrophobicity of strain adhering to solvent was calculated as: % Hydrophobicity = $(1- A_1/A_0) \times 100$

2.6.2. Autoaggregation assay

For autoaggregation assay, overnight grown *Bacillus* sp. SJ-10 cultures at 37 ℃ in nutrient broth was harvested by centrifugation. The collected cells were washed twice with phosphate buffered saline (PBS, pH 7.3) and re-suspended to get absorbance 0.5 at 595 nm. The cell suspension was mixed by vortexing for 10 s followed by incubation at 37℃ for 1 h. Then A_{595} of upper layer was measured. Autoaggregation percentage was expressed as: $1 - (At/A_0) \times 100$, where At represents the absorbance at time t = 1 h and A_0 the absorbance at t = 0.

2.6.3. Enumeration of adhered cell by direct counting

The adhesion abilities of SJ-10 and reference strains were examined using the Caco-2 cell line, as described previously with slight modifications [17]. Briefly, overnight cultured bacteria cell was harvested and then washed 3 times with PBS 100 μL of bacteria (10^7 CFU/mL) was added onto a monolayer of Caco-2 cells in separate wells. The plates were incubated for 4 h at 37℃ in a 5% CO_2 incubator. After incubation, bacterial cells were collected from the wells and Caco-2 cells were washed twice with 1 mL PBS. Adhered cells were treated with 1 mL of 0.5% Triton X-100 for 3 min on ice and then serially diluted with PBS and plated onto MRS agar for quantification.

2.7. Antimicrobial activity

The antimicrobial activities of *Bacillus* sp. SJ-10 was assessed by agar-spot and agar-well diffusion assay [18]. Briefly, in the case of agar-spot assay, the SJ-10 was incubated for 24 h at 37℃ on HM agar plate, and then the colony was subcultured for 18 h in 10 mL of BHI liquid broth. The cultures were centrifuged for 5 min at 10,000 rpm and the pellet resuspended in 2 mL of PBS. The target organisms were grown in appropriate media and culture conditions. For antimicrobial activity testing, 1% of cultured indicator strain was resuspened in BHI liquid broth containing 0.8% agar. The mixture poured in a petri dish and then allowed to solidify under the room temperature. A 5 μL of the SJ-10 inoculum was spotted on the surface, and the dish was incubated at the optimal temperature of the indicator organism for 24 h.

The agar-well diffusion assay as follows; cell-free supernatant (CFS) of the SJ-10 was obtained by centrifugation form subculture as described above. The pH of the supernatants was adjusted to 7.0 with 10N NaOH, and the supernatants were sterile filtered through a 0.2 μm pore-size filter. A 100 μL of indicator was mixed with 10 mL BHI agar in a petri dish. After cooling, 5 mm diameter wells were cut into the agar plates and filled with 10 μL of CFS. The plates were incubated at the optimal condition of the indicators and inhibition zone diameters were measured after appropriate time. The BHI broth was used as negative controls.

2.8. Antioxidants activity

The antioxidant activity of intact cell, CFS and CFE (cell-free extracts) were determined using two methods. The 2,2´-azino-bis (3-ethylbenzthiazoline ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical–scavenging ability assay was performed according to the method of Re et al., 1999 [19]. The Hydroxyl radical antioxidant capacity (HORAC) assay was evaluated by the method described by Hinneburg et al., 2006 [20]. The percentage of ABTS scavenging activity and HORAC was calculated using the following formula: Inhibition (%) = [(Absorbance of the control – Absorbance of the sample)／Absorbance of the control] × 100

2.9. β-Galactosidase activity

β-galactosidase activity was assayed according to the methods of Gilliland and Lara, 1988, and Miller, 1972, with some modification [21, 22]. Each overnight cultured strain was inoculated into MRS supplemented with 1% (v/v) lactose, and grown for 24 h at 37℃ under anaerobic condition. After incubation, the cells was harvested, washed with PBS and re-suspended with 50 mM sodium phosphate buffer (pH 7.5). The suspensions of cell were shaken for 10 min in 4℃ by ultrasonic wave at the setting duty-cycle 30%, output control 3 (Microtip, Branson, Sonifier 250). The 4-nitrophenyl β-D-galactopyranoside (Sigma) as a substrate was added to the tube at a final concentration of 10 μm/mL. The mixture was vortexed and incubated at 37℃ for 10 min. To stop the reaction, 1 volume of 1 M sodium carbonate was added. After the reaction, optical density at both 420 and 560 nm was determined and β-galactosidase activity was calculated as follows: Miller Units = $1000 \times [(OD_{420} - 1.75 \times OD_{550})] / (T \times V \times OD_{600})$

Where OD_{420} and OD_{550} are read from the reaction mixture, OD_{600} is reflects cell density in the washed cell suspension, T is time of the reaction in minutes, V is volume of culture used in the assay in mLs. Finally, Miller Units is defined as micromoles of o-nitrophenol released/min/mL/CFU.

2.10. Phytase activity

The phytase activity was measured by incubating 0.1 mL of enzyme solution with 0.9 mL of 10 mM sodium phytate in 0.1 M Tris–HCl buffer (pH 7.0). The enzyme reaction was carried out at 37℃ for 10 min and then the reaction was stopped by boiling 10 min. The liberated phosphate was measured at OD_{700} after adding 1.5 mL of color reagent, which is prepared freshly before using by mixing four volumes of 1.5% ammounium molybdate solution in 5.5% sulfuric acid and one volume of 2.7% ferrous sulfate solution. One unit of phytase activity was defined as the amount of total enzyme hydrolysing 1 μmol of phosphate per minute under the assay conditions. Protein was determined using Bradford solution with bovine serum albumin as the standard [23].

2.11. Bile salt hydrolase

Bile salt hydrolase activity of the cultures was detected using the plate screening procedure described by Pinto et al., 2006 [24]. Overnight cultures were spotted onto MRS agar plates containing 0.5% (w/v) sodium salt of taurodeoxycholic acid (Sigma) and 0.37g/L $CaCl_2$. Colonies with precipitation zones were considered positive on bile salt hydrolase activity.

2.12. Catalase

The catalase activity was detected by re-suspending the cultures of target bacteria in a 3% solution of hydrogen peroxide.

3. Results and discussion

3.1. Safety assessment for probiotics use of SJ-10

Probiotics strains must be safe for human and animal consumption as the most important point to be considered, and safety assessment was included the lack of harmful activities, such as cytotoxicity and haemolysis against host [25]. A general approach to characterization of a probiotics, to establish guidelines for the safe use, was described by FAO/WHO and these recommendations were [26]: (1) Established history of safe use in traditional products; (2) Absence of a significant risk with regard to transferable antibiotic resistance; (3) Absence of a significant risk with regard to virulence properties; (4) Assessment of certain metabolic activities, such as D-lactate production, bile salt deconjugation; (5) Testing for toxin production if the strain under evaluation belongs to a species that is a known mammalian toxin producer; (6) Determination of hemolytic activity if the strain under evaluation belongs to a species with known hemolytic potential; (7) Assessment of side-effects during human studies; (8) Epidemiological surveillance of adverse incidents in consumers (post-market). In this study on in vitro assessment of potential probiotics candidate SJ-10, cytotoxicity on caco-2 cell, hemolytic activity and antibiotic susceptibility were considered useful for establishing safety by criteria listed above.

The results on safety assessment were shown in Fig. 1. No cytotoxicity to intestinal epithelial cells was found in the SJ-10, with a low percentage of cytotoxicity rates along with *L. plantarum* well known as non-cytotoxic and useful probiotics (Fig. 1A). High toxicity to Caco-2 cells was observed in both pathogenic *B. cereus* and *S. typhi* groups, with significantly increased cytotoxicity compared to that of the SJ-10 and *L. plantarum* groups. Cytotoxicity percentages of *L. plantarum*, SJ-10, *B. cereus*, and *S. typhi* were 7.3%, 9.4%, 72.4%, and 80.3%, respectively. The SJ-10 exhibited non-haemolytic activity without any complete digestion of red blood cell contents surrounding colony and partially lyse (Fig. 1B). It was also susceptible to all tested antibiotics (Table 1). Vulnerability against antibiotics was considered to be best probiotic characteristic,

because pathogenicity and enterotoxin production are closely associated with occurrence of plasmid and it susceptible to each tested antibiotic which ensures its inability to transfer antibiotic resistance as well [27]. The SJ-10 was susceptible to all tested antibiotics in various degrees that is found to be most susceptible for ampicillin (MIC; 15 ng/mL) and less susceptible to streptomycin (MIC; 3000 ng/mL). Additionally, absence of the plasmid was confirmed through electropolation after DNA extraction using alkaline lysis method and then linearization by restriction enzyme digestion (data not shown). In addition to that, in the previous study, a novel *Bacillus* SJ-10, was isolated from *'jeotgal'* derived from squid which is a salted and fermented food in Korean cuisine used as an important additive for improving the taste of foods or alone as a food in itself [23]. Because squid *jeotgal* has a long history more than 1000 years in literature of safe use in food products and did not observed any side effects during the period we have eaten. Therefore, historical aspects can also be proved.

3.2. Resistance to simulated gastrointestinal condition

In order to exhibit their beneficial effects in the host, it is generally accepted that probiotics bacteria must be well alive in the product at the time of consumption and also capable of reaching the large intestine in high enough quantities to facilitate colonization and proliferation [28]. Therefore, acid and bile tolerance must be considered of primary interest, in relation to their germination in the intestinal tract [29, 30]. Changes in viable counts of spores and vegetative cells of SJ-10 in the digestion mixture were monitored during sequential exposure to simulated gastric and intestinal juices.

The Fig. 2 shows the effect of simulated gastrointestinal transit on the spores and vegetative cell viability. In regard to the vegetative cells, inoculation in simulated gastric fluid at 37℃ resulted in a dramatic decline in viable cells, with a 3log reduction after 3 h at pH 2. The number of viable cells remained comparatively constant (10^7 ~ 3×10^7 CFU/mL) during the first 2 h of the simulated gastric phase, and decreased significantly after 3 h to about ~10^5 CFU/mL. Viable cell were

maintained a constant cell number after changed to simulated intestinal condition with ~10^4 CFU/mL (48.4% survivability) up to the end point of in vitro simulated gastrointestinal condition although the initial stage during simulated intestinal condition indicated only temporary damage to vegetative cells due to the low pH stress. In the case of spores, unlike vegetative cell, exhibit no significant variation on viability during the in vitro simulated gastrointestinal fluid. Spore germination rate during continuous culture in the presence of pancreatin and bile salts at pH 8 after 3 h of incubation at gastric condition show > 97%.

Resistance of SJ-10 spores to acid and bile salts with digestive fluid is in agreement with previous reports about several *Bacillus* spp., such as *B. clausii*, *B. subtilis* and *B. licheniformis*, for dietary food supplements, growth promoters and competitive exclusion agents to livestock and enhancement of the growth and disease-resistance of fish in aquaculture [31-34]. Bacterial spores are generated in nature as a means to survive extreme environments by enabling long-time survival in conditions that vegetative cells could not endure. Intrinsic to survival would be the structure of the bacterial endospore, that contains, at its core, a condensed and inactive chromosome. Additional layers surround the spore, including a peptidoglycan-rich cortex and one or more layers of protein material referred to as the spore coat. These protect the spore from exposure to UV radiation, solvents, hydrogen peroxide and digestive enzymes such as lysozyme as well as heat and acidic condition [6]. As demonstrated in some commercial bacillus spores, it suggests that not only the entire dose of ingested SJ-10 spore would be reached the small intestine with intact but also spore could be remained with metabolically stable and well active after its germination during manufacturing and storage conditions as well as intestinal tract of host.

3.3. Adhesion properties

Once probiotics pass through the stomach of acidic condition by maintaining a high viability, and if it has a resistance to the bile salt, adhesion to intestinal epithelial cells is also an essential prerequisite for colonization in the gastrointestinal tract preventing their immediate elimination by

peristalsis and providing a competitive advantage in this ecosystem [35]. To confirm the above precondition, we incubated *Bacillus* sp. SJ-10 with Caco-2 cells under anaerobic conditions and the fraction of adhered cell was quantified by viable cell counts. The results indicated that both spores and vegetative cells of SJ-10 adhered well to monolayer of Caco-2 cells (Fig. 3). Moreover, spores exhibited significantly higher levels of adherence than its vegetative cell, nearly 50% of vegetative cells adhered to monolayers of Caco-2 cells, while nearly 70% of spores adhered to Caco-2 cells. In further analysis in order to gain information to the structural properties of the cell surface of SJ-10, four different solvents were tested: hexadecane, which is a hydrocarbon; xylene, which is an apolar solvent; chloroform, a monopolar and acidic solvent; and ethyl acetate, a monopolar and basic solvent. Bacterial adhesion to hexadecane and xylene correlates with reflects cell surface hydrophobicity. The values of microbial adhesion to solvents obtained with the two other solvents, chloroform and ethyl acetate, were regarded as a measure of electron donor (basic) and electron acceptor (acidic) characteristics of bacteria, respectively [36]. The spore form exhibited the strongly hydrophobic with approximately 82.1% and 67.8% of the spores adhering to hexadecane and xylene, but not for other solvents (Table 2). Whereas, the vegetative cell of SJ-10 showed moderate hydrophobic with 25.6 and 42.3 % adhesion ability to hexadecane and xylene, and more strong affinities to chloroform which means they are strong electron donors.

Moreover, SJ-10 had approximately 2-fold higher autoaggregation ability than all tested reference bacteria. Bacterial autoaggregation is a process whereby bacteria physically interact with each other and settle to the bottom in a static liquid suspension. Thereby, pathogen inhibition is caused through protection against pathogen infection as a natural barrier by probiotics against pathogen exposure in the gastrointestinal tract. In order to assure beneficial effects, probiotics bacteria need to achieve an adequate mass through aggregation (process of reversible accumulation of cells); the aggregation (cell to cell adhesion) of probiotic strains is necessary for adhesion to intestinal epithelial cells [37]. Adherence of bacterial cells is usually related to cell

surface characteristics. Physicochemical characteristics of the cell surface hydrophobicity affect autoaggregation and adhesion of bacteria to different surfaces, and autoaggregation ability and cell surface hydrophobicity are directly correlated [27, 38-41]. Moreover, Del Re et al., 2000, have been reported that strains were able to adhere to cell monolayers if they autoaggregate and manifest a good degree of hydrophobicity as determined by microbial adhesion to hydrocarbons [42]. These previous reports are consistent with our data that SJ-10 show both high hydrophobicity and strong autoggregation ability. Therefore, our results indicated that excellent adhesion to Caco-2 cell and autoaggregation ability of spores and its vegetative cells of SJ-10 would be a good candidate as probiotics.

3.4. Antimicrobial activity

The inhibition effects of *Bacillus* sp. SJ-10 against pathogenic bacteria were shown in Table 3. The SJ-10 showed a broad-spectrum inhibitory activity towards gram-positive as well as gram-negative pathogen, including the *Edwardsiella* spp., which infects fish only (*E. ictaluri*) and occasionally human (*E. tarda*) to cause gastroenteritis and wound infections [43], *Vibrio* spp., which zoonotic pathogen associated with gastroenteritis and cause septicemia [44], *Salmonella typhi*, which cause the typhoid fever and is multi-organ pathogen that inhabits the lympathic tissues of the small intestine, liver, spleen, and bloodstream of infected humans [45], *Shigella sonnei* and *Escherichia coli* O157, which cause acute fever, bloody diarrhea and Hemolytic-uremic syndrome [46], *Aeromonas* spp., which is linked to seafood and water outbreaks, known as human enteropathogens (*A. hydrophila*) and cause the disease known as furunculosis for fish (*A. salmonicida*) [47], *Listeria monocytogenes* and *Bacillus cereus*, which are very important in food safety by causing foodborne illness, and *Streptococcus* spp., which causative agent in the formation of dental cavities in humans (*S. mutants*) and fish pathogen in aquaculture, also can cause opportunistic infections in weakened or immunocompromised humans (*S. iniae*) [48]. The difference was observed according to methods used that gram-negative bacteria were generally

more sensitive, the *Edwardsiella* were most strongly inhibited in agar-spot test, but not in the agar-well diffusion assay. By contrast, regarding gram-positive bacteria, *B. cereus* and *Listeria* strains were not inhibited in the agar-spot test, but their growth was inhibited by the CFS of the SJ-10 in agar-well diffusion assay. These finding that inhibition activity was dependent on the method used, was in line with previous report on bacteriocin like inhibitory substances of *Bacillus amyloliquefaciens* [18]. Moreover, according to Compaore et al., 2013, the different target microorganisms were inhibited by different antimicrobial compounds. Pursuant to this fact, the regulation of their productions by the SJ-10 could be influenced by the target organism and/or the growth conditions in solid or liquid medium. Also it could be explained by instability of the particular antimicrobial compound targeting genus, or that the compound with the activity had not yet been produced at the time of harvesting the CFS used for the test, because production of antimicrobial substances by *Bacillus* is suggested to be under complex genetic regulation [49, 50].

For further insight on potential antimicrobial compound(s) in liquid cultured supernatant, two gram-positive bacteria, *Bacillus cereus* and *Listeria*, were used as indicator organism. CFS of SJ-10 was resistant to all proteolytic enzymes tested and thermal degradation. Regarding the results obtained by the acrylamid gel overlaid with the plate grown the indicator strains after SDS-PAGE of CFS, proteinaceous antimicrobial substances could not detected. Furthermore, the gene involved in lipopeptide production was detected in the SJ-10. Iturin related biosyntasis gene was detected by PCR whereas the bacteriocins (subtilosin A or subtilin) encoding genes were not observed (data not shown). Collectively, gained results suggested that these compounds may be cyclic peptides such as previous observations for *B. amyloliquefaciens* ssp. *plantarum* strains and lipopeptides produced by *B. amyloliquefaciens* LBM 5006 [51-53].

Antibacterial substances produced by different bacteria seem to play an important role in the bacterial antagonism in ecosystems by playing a defensive role to hinder the invasion of ecosystem of other strains or species into an occupied niche. In addition, because bacteriocin

treatment is potentially effective and non-toxic to human and animals, it has been already proposed as an alternative for disease control [54]. The broad inhibitory spectrum of SJ-10 may indicate an ecological advantage, since it would be capable to inhibit several competing bacteria. Of the inhibited pathogens by SJ-10 in this study, particularly, SJ-10 exhibited higher antimicrobial activity against fish related pathogens. Therefore, the antimicrobial substance produced by SJ-10 may represent a significantly strong antimicrobial substance with potential application in the prevention and treatment of zoonoses derived from marine and aquaculture, as well as several food pathogenic and spoilage microorganisms.

3.5. Antioxidant activity

Reactive oxygen species (ROS) are produced as a consequence of the normal metabolism of living organisms. All aerobic organisms including human beings have its own antioxidant defense mechanisms. However, when produced in excess by several factors derived from endogenious and exogenious because this self antioxidant defense system cannot remove all ROS, they can affect biomacromolecules and tissue injury. Thus, one of the key player in colon inflammation is the presence of ROS and cause more than 90% of diseases. Therefore, study for the natural antioxidants source is essentially necessary. In view of this, high antioxidative probiotics would be useful by providing the potential of producing antioxidants during their growth in the intestinal tract.

Hydroxyl radical antioxidant capacity of SJ-10 was estimated because hydroxyl radical is the most reactive oxygen radical and considered the main contributing ROS to oxidation of cellular DNA, protein and lipid [55]. Additionally, total antioxidant activity was also measured using ABTS radical scavenging assay basis of one of the spectrophotometric methods that have been applied as common method to the measurement of the total antioxidant activity [17]. Both hydroxyl radical scavenging and total antioxidant activity were increased in a concentration dependent manner in CFS, and demonstrated higher ability to eliminate the hydroxyl radical than

ABTS radical scavenging, whereas scavenging activity was not observed in intact cell and CFE (Table 4). The CFS possessed the ability to eliminate the hydroxyl radical and ABTS radical at 50% (v/v) concentration by 49.8% and 32.5%, respectively.

It has been shown that extracellular biopolymers, particularly carbohydrate polymers produced by some probiotics, possess antioxidative activity [56-58]. Interestingly, in the case of SJ-10, it was observed its ability to produce an extracellular viscous substance. By physicochemical analysis using FT-IR, NMR, thermal and amino acid analysis, the viscous material was confirmed that poly-γ-glutamic acid (γ-PGA) is a homopolymer composed of glutamic acid residue with sodium salt form. γ-PGA, as one of the representative microbial biopolymer, is a naturally occurring, generally regarded as safe, completely biodegradable, water soluble, edible and nontoxic to human and environment [59]. According to our survey, there was just one report to describe the antioxidant activity of γ-PGA produced by *B. subtilis* D7 isolated from *Doenjang*, a Korean traditional fermented food [60]. The γ-PGA of *B. subtilis* D7 scavenged the ABTS radical in a concentration dependent manner, with maximal reduction of 42.1 ± 1.2 % at 10.0 mg/mL of γ-PGA. Actually, antioxidant research of γ-PGA produced by SJ-10 on cytoprotective effect on hydroxyl radical induced damage to the gastrointestinal tract was conducted, and the paper is currently in press. Therefore, γ-PGA seems to be a major factor for radical scavenging activity.

3.6. Functional enzymatic properties

The SJ-10 exhibited remarkable functional enzymatic activities in β-galactosidase, phytase, bile salt hydrolase and catalase (Fig. 4). β-galactosidase activity of SJ-10 was found to be significantly high as 245.3 U (Miller Unit; micromoles of o-nitrophenol released/min/ml/CFU), and in the following order; LF (211.8 U), LC (152.7 U), LR (114.5 U) and LP (60.5 U) (Fig. 4A). In our previous study, it was characterized that BglC, one of the four types identified β-glucosidases in SJ-10, is broad substrate specific β-glycosidase with high activity against β-galctoside as well as β-glucoside substrates [61]. For this reason, due to broad substrate specificity, SJ-10 may have a

high activity in β-glactosidase activity. Gut microbial β-glycosidase could relieve lactose maldigestion symptom (also called lactose intolerance) causing stomachache with diarrhea in widespread human suffering by hydrolyzing lactose to glucose and galactose [62]. Moreover, it could carry out biotransformation of various organic matters, such as soybean isoflavones, ingested with food into the bioactive molecules that positive effect on host health. The hydrolysis of soybean isoflavones generate functional aglycone which daidzein from daidzin or genistein from genistin, and then by the gut microbiota these being transformed sequentially into S-equol which known as most effective in stimulating an estrogenic response among the isoflavone derivatives [63-65]. Therefore, probiotic candidate bacteria having a sufficiently high β-galactosidase activity contribute to the potential health promotion benefits, and it can enough expected these effects to *Bacillus* sp. SJ-10.

The phytase activity of SJ-10 was even significantly higher than *L. plantarum* which is known as phytate degrading LAB used to enhance the mineral solubilization of cereal-based products (Fig. 4B) [66, 67]. A phytate (myo-inositol hexakisphosphate) is related with dietary fibre, naturally present in various vegetable foods, in particular legumes and whole grains. It is considered as anti-nutrient factor in regard to nutrition by strong binding affinity to important metal ions such as calcium, magnesium and zinc. Thereby its complex forms lead to hinder the bioavailability of essential minerals [68]. Therefore, hydrolysis of phosphate groups in phytate promote mineral uptake on host, and increase the nutritional quality of phytate-rich foods and feeds [69-71]. In view of improving digestibility and reducing anti-nutritive effects, the better phytase activity of SJ-10 suggests a potential useful functionality as probiotics.

A Fig. 4C showed the bile salt hydrolase (BSH) and catalase activity of SJ-10. The precise functions of BSH in probiotics bacteria itself is currently unknown although several hypotheses have been proposed such as bile detoxification, gastrointestinal persistence, nutritional role and membrane alterations [72]. Nevertheless, a number of studies on impact to the host so far strongly

support that BSHs function in probiotics strains is detoxification of bile salts. Bile detoxification maximize the survivability of target probiotics in the hostile environment of intestinal tract, thereby increased persistence is able to induce the overall beneficial effects related to the strain [73-74]. A great part of the unconjugated bile acid generated from hydrolysis of conjugated bile acid by BSH is precipitated and excreted in feces. Likewise, white opaque precipitations of SJ-10 colonies were detected through the in vitro evaluation using plate assay. Furthermore, this ability to precipitate has been suggested that associated with cholesterol lowering because deconjugation is bring to promote the de novo synthesis of cholesterol and thereby decreased serum cholesterol.

The SJ-10 was catalase-positive equally other *Bacillus* spp., as efficient producer of catalase, whereas in some human and animal microbiota, *Lactobacillus*, *Bifidobacteria*, *Streptococcus* spp., lacking catalase [75]. Microbial catalase obviously can alleviate the undesirable effects of free radicals on host by decomposing excessively produced hydrogen peroxide to water and oxygen [76]. Hydrogen peroxide could be more harmful to biological system than other ROS since enough time to travel into the cell nucleus by its relatively low reactivity. It can wreak havoc on macromolecules such as DNA, protein and lipid [77]. Thus, the removing of hydrogen peroxide generated during metabolic process is very important for antioxidant defense. In this sense, if potent catalase-positive probiotics strain, not existing in LAB, is inhabited in the intestinal tract could act as powerful antioxidants.

3.7. Utilization of prebiotics

In order to re-establish the bacterial homeostasis, one major approach is to employ the use of prebiotics [78]. Because non-digestable oligosaccharides and carbohydrate, such as inulin, fructo-oligosaccharides (FOS) and β-glucan, are not digested by human and animals enzymes, these can be added to foods and feeds as dietary fibers, and can be used as prebiotics enable to stimulate the selective growth of certain bacteria strains. Particularly, inulin and FOS had been demonstrated prebiotics properties and health benefits, and already recognized as the well established

supplements, and the mainly products sold in the world-wide market are non-digestible oligosaccharides like those.

Meanwhile, nowadays several investigations have been focused on non-digestible long chain complex carbohydrates for their potential as novel prebiotics [79]. Cereal β-glucans (in particular derived from oat and barley) have been demonstrated not only health promoting effects that cumulative evidence obtained from animal models and human intervention studies strongly suggest positive effects such as immuno-stimulatory/modulating effect, pathogen inhibition, anti-tumor, anti-cancer, cholesterol lowering capacity, inhibiting atherosclerosis, but also the potential as a novel source of prebiotics due to their competency to pass undigested through the alimentary canal, where they act as a selective substrate for stimulate the growth and activity of a small number of certain beneficial bacteria [80].

SJ-10 was shown a good growth similar to glucose when FOS, inulin or barley β-glucan as sole carbon source (data not shown), additionally, in the case of substrate β-glucan, its hydrolysate and uptake pattern by SJ-10 was detected using TLC (Fig. 5). The β-glucan was hydrolyzed into the cellotriose and cellotetraose (after 12h fermentation), and simultaneously absorb cellotriose first by SJ-10, and continuously tetraose was also uptaken. As a result, both cellotriose and cellotetraose was completely consumed at 72 h. Oligosaccharides are more selectively fermented by probiotics than their high-molecular mass counterparts. This property would be owing to the fact that the low-molecular substrates have more non-reducing ends per unit mass, which favors a more rapid attack by the exo-acting enzymes produced by probiotics organisms [81]. However, the detailed mechanism on bacterial selective fermentation of β-glucans remains unclear, and there are many research challenges for further study in this area. In our previous study, a novel β-glucanase of SJ-10 was characterized and confirmed that the barley β-glucan was degraded into triose and tetraose by this enzyme [82]. Same degradation pattern was also shown in this study. Therefore, β-glucanase of SJ-10 would be major role in barley β-glucan fermentation.

4. Conclusion

Although in vitro assessment of the functionality of potential probiotics candidate has its limitation, such as insufficient a gastrointestinal environment and deficient interactions with the intestinal microbiota as well as host, rapidly and precisely obtained information allows to assess the potential of tested probiotics candidate and suggest future research directions effectively. We assessed the potential probiotics effects of SJ-10 in vitro condition. To summarize the obtained results, the SJ-10 isolated from fermented seafood was shown that non-totoxic against human epithelial colorectal adenocarcinoma cell and red blood cell, acid and bile tolerance of its spores, strong adhesion and autoaggregation capacity, broad spectrum antimicrobial effect, hydroxyl radical scavenging antioxidant activity, functional enzymatic properties and utilization of barley β-glucan as well as FOS and inulin. In conclusion, the marine-derived *Bacillus* sp. SJ-10 demonstrate potential for use as food and feed supplementation to human and animals, particularly it could be applied directly as a promising alternative to antibiotics and health enhancing agents for use as a feed additive in aquaculture. Furthermore, in vivo test is currently in progress.

Table and Figure

Table 1. Antibiotic susceptibility of *Bacillus* sp. SJ-10 and MIC values

Antibiotics	MIC (ng/mL)	S / I / R[a]
Streptomycin	3000	S
Kanamycin	300	S
Cabenicillin	300	S
Tetracycline	150	S
Chloramphenicol	102	S
Penicillin	60	S
Ampicillin	15	S

[a] Susceptibility categories: S, susceptible; I, intermediate; R, resistant

Table 2. Cell surface hydrophobicity and autoaggregation

Bacteria	Hydrophobiciy (%)				Auto-aggregation (%)
	Hexadecane	Xylene	Chloroform	Ethyl acetate	
LC	18.1 ± 0.8	34.7 ± 1.7	39.8 ± 2.0	14.2 ± 0.6	10.2 ± 0.5
LF	8.5 ± 0.3	10.8 ± 0.6	4.9 ± 0.2	< 1.0	8.3 ± 0.4
LP	9.2 ± 0.5	49.7 ± 2.1	69.1 ± 3.5	24.4 ±1.2	12.1 ± 0.6
LR	7.8 ± 0.4	16.2 ± 0.7	37.2 ± 1.9	< 1.0	9.2 ± 0.5
SJ (Vegetative cells)	63.3 ± 3.1	59.0 ± 2.4	11.2 ± 0.6	17.6 ± 1.0	45.1 ± 2.3
SJ (Spores)	82.1 ± 3.8	67.8 ± 3.4	4.7 ± 0.2	5.2 ± 0.1	-

LC; *Lactobacillus casei*, LF; *L. fermentum* KCTC 13097, LP; *L. plantarum* ATCC 14917, LR; *L. rhamnosus* ATCC 7469, SJ; *Bacillus* sp. SJ-10

Table 3. Antimicrobial activity of SJ-10 against the tested pathogenic bacteria by agar spot (*) and agar well diffusion test (**).

Indicator strain and source	Antimicrobial activity	
Gram negative	*	**
Edwardsiella ictaluri ATCC 33202	+++	+
E. tarda ATCC 12267	+++	+
Vibrio anguillarum O1	-	-
V. alginolyticus KCTC 2472	+	+
V. cholerae	++	+
V. parahaemolyticus KCTC 2471	++	+
V. vulnificus KCTC 2982	-	-
Salmonella typhi	+	-
Shigella flexneri	-	-
S. Sonnei	+	-
Escherichia coli O157	+	+
Aeromonas hydrophila	+	-
Gram positive		
Listeria monocytogenes KCTC 3071	-	+
L. monocytogenes KCTC 13064	-	+
Bacillus cereus KCTC 3624	+	++
Streptococcus iniae KCTC 3657	+	+
S. mutans KCTC 3065	-	-
Staphylococcus aureus subsp. *aureus*	-	+
Lactococcus garvieae KCTC 3772	-	+

-; no inhibition, +; 1 ≤ inhibition < 10mm, ++; 10 < inhibition ≤ 20mm, +++; 20 < inhibition.

Table 4. Antioxidants activity of *Bacillus* sp. SJ-10.

Conc (v/v)	Scavenging rate of Hydroxyl radicals (%)			Scavenging rate of ABTS+ radicals (%)		
	Intact cell	CFS	CFE	Intact cell	CFS	CFE
10 %	0.3 ± 0.1	12.2 ± 0.6	1.3 ± 0.1	0.2 ± 0.0	7.3 ± 0.4	0.7 ± 0.0
20 %	0.3 ± 0.1	26.8 ± 1.3	2.1 ± 0.1	0.2 ± 0.0	15.1 ± 1.8	1.2 ± 0.0
30 %	0.5 ± 0.3	38.2 ± 1.9	2.4 ± 0.1	0.3 ± 0.1	23.6 ± 2.3	2.4 ± 0.1
40 %	0.4 ± 0.2	46.3 ± 2.3	3.6 ± 0.2	0.2 ± 0.1	28.9 ± 2.2	2.8 ± 0.1
50 %	0.6 ± 0.3	49.8 ± 2.4	3.8 ± 0.2	0.3 ± 0.1	32.5 ± 1.5	3.1 ± 0.2

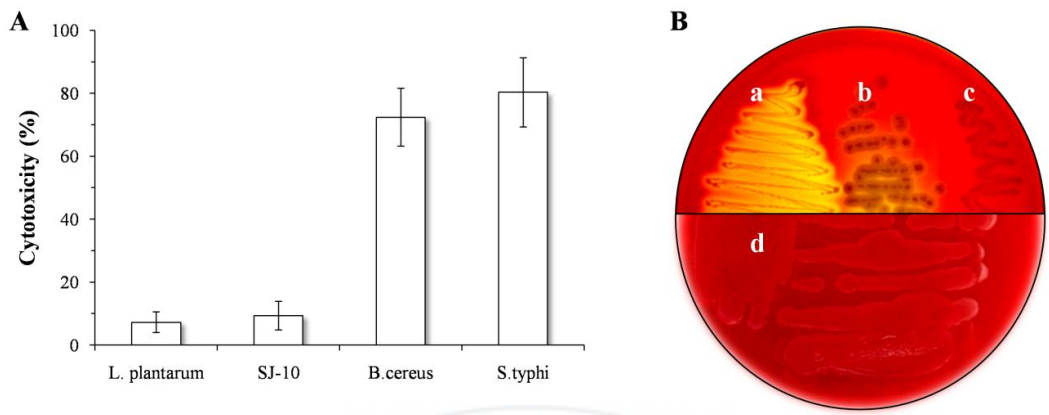
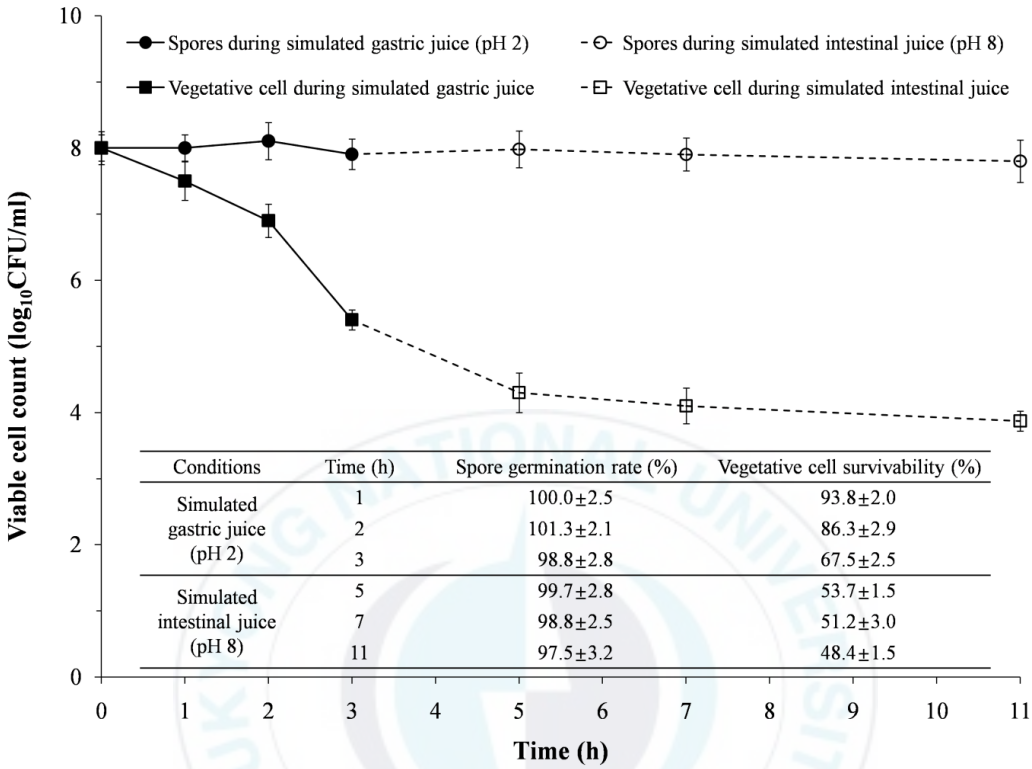


Fig. 1. Safe assessment of *Bacillus* sp. SJ-10. (A) Cytotoxicity on Caco-2 cells of SJ-10 compared with probiotics and pathogenic strains (B) Hemolytic activity on sheep blood agar. a; β-hemolysis, b; α-hemolysis, c; γ–hemolysis and d; non-hemolysis of SJ-10.



Conditions	Time (h)	Spore germination rate (%)	Vegetative cell survivability (%)
Simulated gastric juice (pH 2)	1	100.0±2.5	93.8±2.0
	2	101.3±2.1	86.3±2.9
	3	98.8±2.8	67.5±2.5
Simulated intestinal juice (pH 8)	5	99.7±2.8	53.7±1.5
	7	98.8±2.5	51.2±3.0
	11	97.5±3.2	48.4±1.5

Fig. 2. Acid and bile tolerance of SJ-10 during simulated gastrointestinal juice.

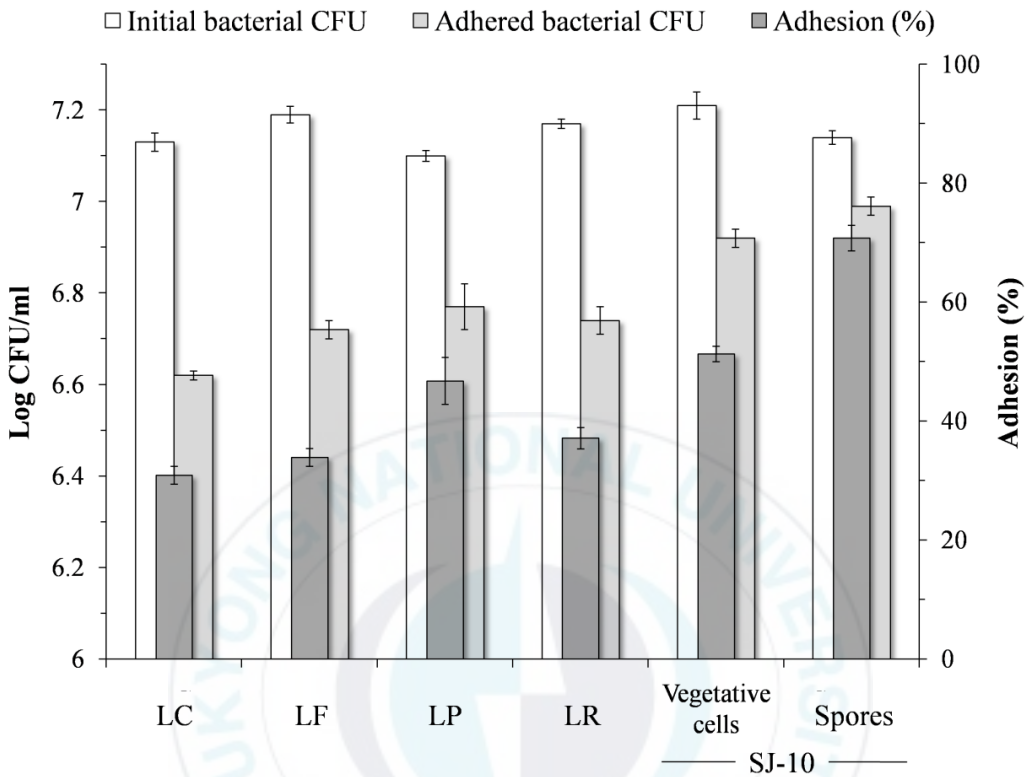


Fig. 3. Bacterial adhesion to Caco-2 cells. The adherence capability of each strain was compared by plate counting between initial and adhered bacterial CFU. LC; *Lactobacillus casei*, LF; *L. fermentum* KCTC 13097, LP; *L. plantarum* ATCC 14917, LR; *L. rhamnosus* ATCC 7469, SJ; *Bacillus* sp. SJ-10

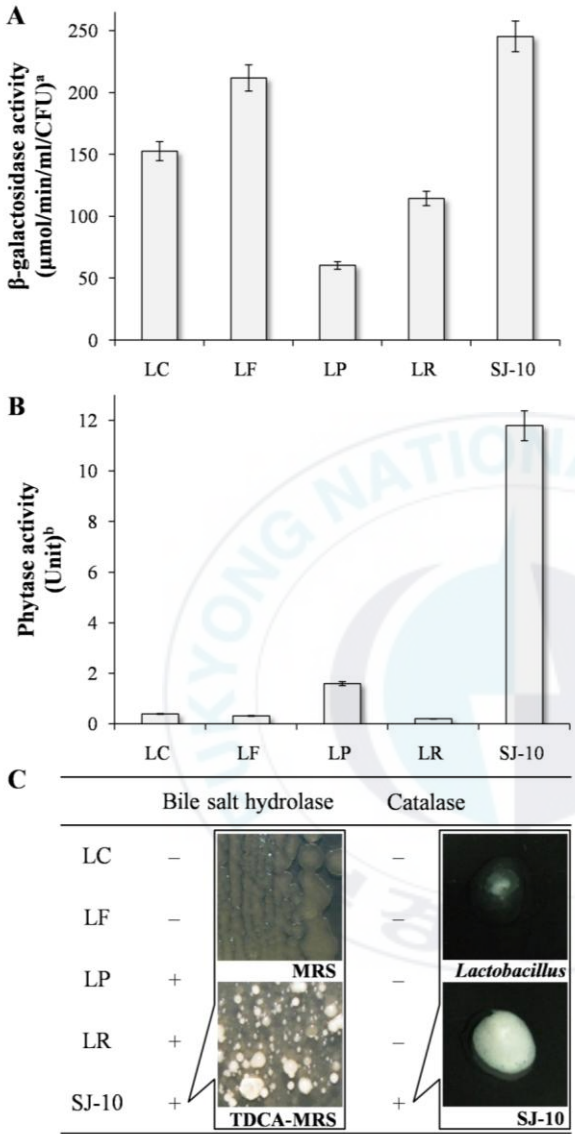


Fig. 4. Functional enzymatic properties. β-galactosidase (A), phytase (B), bile salt hydrolase and catalase (C) of SJ-10 and reference strains. LC; *Lactobacillus casei*, LF; *L. fermentum* KCTC 13097, LP; *L. plantarum* ATCC 14917, LR; *L. rhamnosus* ATCC 7469, SJ; *Bacillus* sp. SJ-10

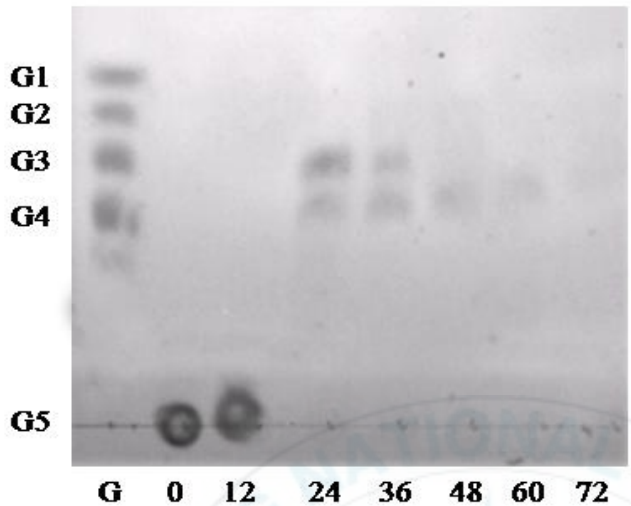


Fig. 5. Thin-layer chromatography of degraded products during fermentation of barley β-glucan by SJ-10. Lane G; G1: glucose, G2: cellobiose, G3: cellotriose and G4: cellotetraose, Lane 0-72; fermentation time (0-72 h)

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Chapter 2

Physicochemical properties, production and biological functionality of poly-γ-glutamic acid with constant molecular weight from halotolerant *Bacillus* sp. SJ-10

Abstract

In this study, extracellular viscous biopolymer produced by halotolerant *Bacillus* sp. SJ-10 isolated from traditional Korean salted-fermented seafood, *Ojingeo-jeotgal* (sliced squid), was purified and characterized. By physicochemical analysis using FT-IR, NMR, TGA and TLC analysis, the biopolymer was proved as a γ-PGA composed of only glutamic acid residue with sodium salt form. When batch fermentation was carried out with 3% sucrose, 1% NH_4Cl, 3% L-glutamic acid and 8% NaCl in 37°C at pH 7 for 3 days, the SJ-10 produced about 24.7 g/L γ-PGA with constant molecular weight as ~400kDa, and regardless of the molecular weight, the maximum yield was 26.2 g/L in 6% NaCl fermentation condition. The sodium salt γ-PGA with molecular weight of 400kDa was found to have total antioxidant activity by scavenging the ABTS+ radical and reducing molybdenum, with maximal scavenging activity at 0.5 mg/mL and reduction capability at 1 mg/mL (20 μg ascorbic acid equivalent), respectively. These results suggest potential availability in the food, cosmetic and biomedical industries for the γ-PGA having certain quality as natural antioxidant as well as provide an economical method for controlling the molecular weight.

Keywords; Poly-γ-glutamic acid, Halotolerant *Bacillus*, Constants molecular weight, Antioxidant

1. Introduction

Poly glutamate as an unusual anionic homopolyamide is made of L- and/or D-glutamic acid units [1]. It can be classified two isoforms, poly-α-glutamic acid (α-PGA) and poly-γ-glutamic acid (γ-PGA), according to the attachment of carboxy group. Although α-PGA can be produced by recombinant technology since the difficulty for production using the naturally occurring biological systems of microorganism, commonly, it is artificially synthesized by nucleophile-initiated polymerization of the γ-protected N-carboxyanhydride of L-glutamic acid. In the case of chemically synthesis, the molecular weight of products has less than 10 kDa which are used to a limited extent such as a surface-modified material [2].

Meanwhile, γ-PGA, as one of the representative microbial biopolymer, is a biodegradable, edible and nontoxic to human and enviroment thereby generally regarded as safe. It can be consist of not only L- or D-glutamic acid residues but also both L- and D-glutamic acid residues, which is synthesized in a ribosome-independent manner and polymerized by the γ-amide linkages in the cell [3]. Hence, unlike other homopolyamides such as poly-L-lysine and cyanophycin, γ-PGA has the unique properties of protease resistance, various molecular mass and wide-range industrial applications [4]. Additionally, γ-PGA exists in the aqueous solution as a water-insoluble free acid form or its salt form combined with various cations (Na^+, Mg^{2+}, K^+, NH^{4+} or Ca^{2+}), which is completely water-soluble [5]. The molecular mass of γ-PGA produced from bacteria has over than ~10 kDa and commonly ranges from ~100 to 1,000 kDa. Based on its molecular weight, γ-PGA has been widely used as biopolymer flocculants (6,200, 5,800and 2,000 kDa) [6-8], tissue engineering materials (2,000 and 1,250 kDa) [9, 10], heavy metal and dye removal (2,500~990 kDa) [11-15], probiotics protectant (257 kDa) [16, 17], drug carrier/deliver (45~60 kDa) [18, 19], and calcium absorption (11kDa) [20, 21]. Although γ-PGA has wide variety of application, the high production cost is currently major limitation to routinely use in industrial fields. Therefore, to achieve production of high quantity and quality of γ-PGA which have proper certain molecular

weight for industrial application, many researchers have been efforts to isolate desirable γ-PGA-producing bacteria, determine culture conditions and optimize media compositions.

γ-PGA producers are known as a few gram-positive bacteria (*Bacillus* spp., *Staphylococcus* spp., etc), archaea (*Natrialba*) and eukaryotic (*Saccharomyces cerevisiae*) microorganisms. Among them, gram-positive bacteria mainly the *Bacillus* spp. has been attracted attention as a most desirable γ-PGA producer because of its promising production yields, availability of various nutrients source and in-depth understanding of the metabolic processes. Furthermore, γ-PGA which polymerized by *Bacillus* have diverse biochemical function enabling to play different roles depending on the each strain according to their habitat, like that secreted γ-PGA in high salinity environments act as a cytoprotection [4]. In the previous our study, a bacterium was isolated from Korean traditional salted-fermented seafood. The bacterium was identified as a novel species, *Bacillus* sp. SJ-10, based on their biochemical characteristics and phylogenetic analysis using 16S rRNA sequence [22]. The present study has described physicochemical properties and biofunctionality of γ-PGA produced by *Bacillus* sp. SJ-10, furthermore, advantageous characteristics of this strain as γ-PGA producer were illustrated.

2. Material and methods

2.1. Microorganism

The strain SJ-10 has been deposited in Korean Culture Center of Microorganisms (KCCM) and Japan Collection of Microorganisms (JCM) with accession number KCCM No. 90078 and JCM No.15709, respectively. The bacterium was maintained on agar plates of halophilic media (HM; 0.1% glucose, 0.5% yeast extract, 0.5% proteose peptone and 5% NaCl) and stored at 4°C, while stock cultures were maintained in cryoprotectants at -70°C.

2.2. Isolation of γ-PGA

The bacterial stock was spread onto the HM agar plate and then incubated in 37°C standing incubator. After overnight incubation, the single colony was picked up and inoculated into 10 ml HM broth in a 50 mL test tube, followed by overnight cultivation in shaking incubator at 37°C. After cultivation, the seed culture (1% v/v) was inoculated into 100 mL fermentation medium consist of glucose (10 g/L), NH_4Cl (5 g/L), sodium chloride (50 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $CaCl_2 \cdot 2H_2O$ (0.15 g/L), $MnSO_4 \cdot 4H_2O$ (0.15 g/L) and $FeCl_3 \cdot 7H_2O$ (0.04 g/L) in 500 mL Erlenmeyer flask. After fermentation for 3 days, the viscous culture broth was separated to remove the cells and solid particles by centrifugation at 10,000 rpm for 10 min. The collected supernatant was thoroughly mixed with three volumes absolute ice-cold ethanol, and then the mixture was stored at 4°C. The resultant precipitates were recovered by centrifugation at 12,000 rpm for 20 min, dissolved in appropriate volume of distilled water, boiling for 10min and then centrifuged to remove any insoluble contaminants. The aqueous γ-PGA was desalted by dialysis and lyophilized. The lyophilized powder form γ-PGA was used for further analyses.

2.3. Physicochemical characterization of γ-PGA

2.3.1. Fourier transform infrared (FT-IR) spectra

The purified γ-PGA was used for FT-IR measurements in the frequency range of 4000–650 cm^{-1} using Perkin Elmer (USA), Spectrum X. The γ-PGA was placed at the surface of the ATR crystal,

and a pressure was applied to expel air from the powder particles. The spectrum of the sample was collected by averaging 128 scans at four resolutions.

2.3.2. Nuclear magnetic resonance (NMR) spectroscopy

Measurement of proton (^{1}H)-NMR and carbon (^{13}C)-NMR spectra were performed with a JEOL (Japan), JNM ECP-400 NMR spectrometer at 400 and 100 MHz, respectably. ^{1}H-NMR chemical shifts in parts per million (ppm) are reported using HDO at 4.8 ppm as an internal reference. The concentration of the γ-PGA sample and its derivatives are 1.0% (w/v) in D_2O solutions. The pH of samples was adjusted by adding aliquots of concentrated NaOD solutions. The pH values are direct meter readings of a digital pH meter without correction for deuterium effects. The temperature was 30 ℃. Peak areas for ^{1}H-NMR spectra were measured by digitally integrating and are reported as relative peak areas representing a given number of hydrogens. The chemical shift of 1H spectra was measured relative to the 3-(trimethylsily) propionic acid-d_4 sodium salt (TSP) signal as 0 ppm.

Measurement of ^{13}C-NMR spectra was performed with a JEOL, JNM ECP-400 at 100 MHz in 10 mm tubes. The concentration of γ-PGA samples was 1.0% (w/v) in D_2O solutions, the temperature was 30℃, and concentrated NaOD solutions were used to adjust pH values. The chemical shift of ^{13}C spectra was measured relative to the dioxane signal from 76.8 ppm.

2.3.3. Thermal analysis

Thermal degradation temperature (T_d) was determined by thermal gravimetric analysis (TGA). The purified γ-PGA powder weights of about 15 mg were used in dynamic experiments. TGA were implemented using a PERKIN-ELMER (USA), at a heating rate of 10℃·min^{-1} under nitrogen atmosphere flow of 25 mL·min^{-1}, in temperature range of 50-700℃.

2.3.4. Amino acid composition

The purified γ-PGA was hydrolyzed with 6N HCl at 110℃ for 6 h in a sealed and evacuated tube for amino acid analysis. Thin-layer chromatography was performed on a cellulose plate (Merck,

USA) with solvent systems of butanol/acetic acid/water (3:1:1, w/w) and 96% ethanol/water (63:37, w/w). Amino acids were detected by spraying with 0.2% ninhydrin in acetone.

2.3.5. Methylene blue staining

In order to estimate the approximate molecular size and degree of polymerization, SDS-PAGE was done for visualization of γ-PGA using methylene blue [23]. Portions of purified γ-PGA, 10 μL (1mg/mL) were loaded on 6% polyacrylamide gel. A commercial protein ladder marker (Enzynomics, Korea) was used as a molecular weight standard. The proteins were stained with coomassie brilliant blue and de-stained with 7% acetic acid in 10% methanol. And then the gel was re-stained for γ-PGA with 0.5% methylene blue in 3% acetic acid and de-stained with distilled-water.

2.3.6. Molecular weight determination

The molecular weight of γ-PGA was estimated by gel filtration. It was accomplished by comparing the effluent volume of the γ-PGA and dextrans from a Sephadex G-100 column. The Sephadex G-100 column (2 × 50cm) was equilibrated with phosphate buffered saline (PBS). After 10 mg of lyophilized γ-PGA was dissolved completely in 2 mL PBS, the solution was applied to the top of the column. And then it was eluted using PBS at a flow rate of 1.3 mL/min at room temperature (18-20℃). Each eluted fraction (1 mL) was collected and estimated spectrophotometrically at 280 nm. Molecular weight of γ-PGA was calculated by substituting the fraction number using a calibration curve computed by dextrans standard molecular weight of 580,000, 71,000 and 11,000. Partition coefficients (Kav) were calculated as Kav = (Ve - Vo) / (VT - V0) (Ve, elution volume; Vo, void volume; Vt, total volume). The void volume of the column was determined using blue dextran with molecular weight of 2,000,000 Da.

2.4. Optimization of fermentation conditions for γ-PGA production

To evaluate the effect of fermentation conditions and media components on γ-PGA production from *Bacillus* sp. SJ-10, fermentation was performed at different temperature (4-55℃), pH (5-11),

L-glutamic acid (0-5%) and NaCl concentrations (0-16%). The tested carbon sources were glucose, sucrose, starch, galactose, glycerol, maltose, mannose, xylose, manitol, lactose and fructose, and used at a final concentration of 3% w/v. The tested nitrogen sources were NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$, yeast extract, L-alginine and peptone. Each nitrogen source was added at a final concentration of 1% w/v. L-glutamic acid, NaCl, C- and N- source were used by adding or replacing in the isolation medium. After inoculation of seed culture (2% v/v) into erlenmeyer flasks containing 1 L of fermentation medium, they were incubated in different conditions at 150 rpm. During the fermentation, samples were withdrawn periodically for investigation of production yield.

2.5. Total antioxidant activity

Total antioxidant activity of the γ-PGA was determined by measuring the ability scavenging 2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radicals and reducing the phosphomolybdenum [24, 25]. The ABTS radical solution was prepared by mixing with same volume of 7 mM ABTS and 2.45 mM potassium persulfate in water, and then the mixture was put into the darkroom for 12 h. The ABTS radical scavenging activity was carried out by reaction of 900 μL of ABTS radical solution and 100 μL of sample at various concentrations. The absorbance was measured at 734 nm against a blank. Percentage of ABTS scavenging activities were computed by the following equation: Inhibition (%) = [(Absorbance of the control – Absorbance of the sample)／Absorbance of the control] × 100

The phosphomolybdenum assay was evaluated that an aliquot of 100 μl at various concentrations of γ-PGA is reacted with 900 μL reagent solution composed of 4 mM ammonium molybdate and 28 mM sodium phosphate in 0.6 M sulphuric acid. And then the reaction mixtures were incubated in boiling water for 90 min. After incubation and cooling, the absorbance was measured at 695 nm against a blank in which the same amount of water instead of the γ-PGA. The antioxidant activity using phosphomolybdenum method was expressed as ascorbic acid (AA) equivalent.

3. Results and discussion

3.1. Purification of γ-PGA

The SJ-10 was observed primarily its ability to produce an extracellular biopolymer considered as γ-PGA. This property was observed by mucoid colony morphology on HM solid medium (Fig. 1A, B). To physiological and chemical characteristic analysis of the extracellular polymer, after fermentation 3 days at 37℃ in fermentation media, it was precipitated form cell free supernatant using ethanol. Subsequently, proteins were removed, dialysed and lyophilized. As a result, a white powder was obtained (Fig. 1C).

3.2. Physicochemical characteristics

3.2.1. FT-IR and NMR spectroscopy

The chemical composition of the purified extracellular polymer synthesized by the *Bacillus* sp. SJ-10 was analyzed by FT-IR and NMR spectroscopy (Fig. 2). The FT-IR absorption bands were shown in Fig. 2A., with four major peaks the absence of strong peak at 1750 cm^{-1} representing Carbonyl group: Amide I, N-H bending band at 1627 cm^{-1}; Amide II, stretching band at 1558 cm^{-1}; and C=O symmetric stretching band at 1402 cm^{-1}. Additionally, the characteristic peak observed at 2500 – 3600 cm^{-1} with broad band, overlap of O-H, N-H and C-H stretching vibrations. These appearance depicted typical peaks of γ-PGA. Moreover, it was revealed that specific two adjacent peaks, at about 1620 – 1655 cm^{-1} and 1550 – 1585 cm^{-1}, which are exhibited only pure sodium salt γ-PGA (Na^+ form) coincide with previous reports [26].

Additionally, we analyzed the ^1H- and ^{13}C-NMR spectrum to further insight of chemical composition (Fig. 2B, C). ^1H-NMR for γ-PGA in D_2O showed that chemical shifts are: 3.66 ppm for α-CH proton; 2.08 ppm for β-CH_2 proton; and 2.33 ppm for γ-CH_2 proton. Additionally, the chemical shifts of ^{13}C-NMR were: 55.53 ppm for α-CH_2 group; 27.77 ppm for β-CH_2 group; 34.34 ppm for γ-CH_2 group; 175.21 ppm for CO group; and 181.96 ppm for COO^- group. Likewise with FT-IR results, these NMR spectra were consistent with the occurrence of sodium

salt γ-PGA reported by Ho et al., 2006 [27]. To sum up, viscous biopolymer produced by SJ-10 was confirmed to sodium salt γ-PGA.

3.2.2. Thermal analysis

TGA was performed to determine the T_d which represents thermal stability of γ-PGA. The percentage of weight loss of dried γ-PGA was analyzed while increasing the temperature from 50 to 700℃. Fig. 3 was shown thermal gravimetric curve. The thermal decomposition temperature of γ-PGA was 310℃ by occurring significantly decrease of the molecular weight at that temperature, and the temperature which half of initial weight was 450℃. Thermal degradation of γ-PGA is generated by cyclodepolymerisation [28]. The glutamic acid residue existed in the end of polymer is converted into the pyro-glutamic acid which cyclized form of glutamic acid by heat. At that instant the pyro-glutamic acid is released from the polymer thereby gradually decreasing the molecular weight. In this thermal analysis result indicate that γ-PGA has high resistant to thermal degradation and suggest for application wherein processes requiring high temperature.

3.2.3. Amino acids and enantiomeric composition analysis

Thin-layer chromatography was performed for analyzing the amino acid composition of γ-PGA from *Bacillus* sp. SJ-10. The spot of hydrolysate was observed at a position corresponding to L-glutamic acid with same Rf values (Fig. 4). Meanwhile, ninhydrin reaction of purified γ-PGA itself was not shown any spot. It demonstrated that the peptide bond of γ-PGA is γ-linked bond. From these above results, the isolated biopolymer was found to be a γ-PGA consisted of only glutamic acid.

3.3. Effect of NaCl concentration on the molecular weight of γ-PGA

The NaCl effects against molecular weight and productivity during γ-PGA fermentation was checked. By the SDS-PAGE using methylene blue staining, we confirmed the apparent molecular weight after fermentation in the medium containing various concentrations of NaCl. Fig. 5A showed the effect of NaCl concentration on the molecular sizes of γ-PGA (The results on γ-PGA

yields according to additional NaCl are presented in section 3.4.3). γ-PGA was exhibited as smear bands in the range of 0-6% NaCl containing media. However, the clear band was observed when the NaCl concentrations increased over 8%. Therefore, it showed constant molecular weight γ-PGA in the increased salt concentration.

Regulatory effects of NaCl on γ-PGA molecular weight were reported from several halotolerant *Bacillus* strain. The γ-PGA produced from *B. megaterium* with a greater molecular size was obtained from a medium containing a higher salt concentration [29]. The average molecular masses of the polymer in 5 and 10% NaCl containing media were estimated to be ~1,000 and >2,000 kDa, respectively. In the case of *B. subtilis* (chungkookjang), γ-PGA with quite high molecular sizes (over than 1,000 kDa) were synthesized in media containing low NaCl concentrations (0.05 and 0.5%) [30]. In contrast, under high saline conditions (10 and 25%), γ-PGA of comparatively low molecular size (10 ~ 200 kDa) were produced. *B. licheniformis* WX-02 also showed the effect of NaCl concentration on the molecular weights of γ-PGA [31]. The molecular weight variation range was higher in the presence of NaCl compared with absence of NaCl, moreover, molecular weight decreased when the NaCl concentrations increased (0 ~ 10%). Nevertheless, γ-PGAs produced these *Bacillus* strains which decreased or increased with molecular range variation by the salt were quite different to that of the *Bacillus* sp. SJ-10 because the molecular weight is become constant when the increased NaCl concentrations.

Molecular weight of γ-PGA produced in 8% NaCl was detected by gel filtration with dextrans standards. It was determined to be ~400kDa with no significant variation of molecular weight range as shown in Fig. 5B. Molecular characterization of γ-PGA is essential in order to evaluate its properties and hence its potential areas of application. High molecular weight γ-PGA (>106 kDa) is desirable for the majority of applications as a viscosity-adding agent. Over the 1,000 kDa of γ-PGA is more proper for the flocculants, tissue engineering materials and heavy metal and dye removal. In contrast, antifreeze activity and calcium absorption ability of γ-PGA are increased

with the decreased its molecular weight (<20 kDa), moreover, γ-PGA with low molecular weight (30 ~ 50 kDa) is required for drug delivery applications. Therefore, different molecular sizes of γ-PGA were required for different purposes. Although γ-PGA has wide variety of application, the cost of production is presently very high and this is a major limitation to the widespread application of the polymer. Thus, to obtain high quality and quantity of γ-PGA with certain molecular weight for industrial application, many researchers have been exerted their efforts to screen high γ-PGA producing bacteria, develop of recombinant host using metabolic engineering, determine culture conditions and optimize media compositions [5]. In terms of economical mass production, simple method that can produce a certain molecular weight using NaCl can be exerted synergistic effect with the above efforts.

In general, *Bacillus* spp. abundantly produces extracellular enzymes, γ-PGA depolymerase is also one of them during γ-PGA production, and this enzyme hydrolyze the produced γ-PGA [32]. Therefore, the molecular sizes of γ-PGAs are variable. In the case of SJ-10, however, the activity of γ-PGA depolymerase could be no active or significantly low at high salinity condition, and thereby the γ-PGA having constant molecular weight is produced. Additionally, it may cannot even proper active against γ-PGA produced by itself due to the structural feature by L/D glutamic acid ratio. Although further research must be conducted to prove such a conclusion, the data at least indicate that it is possible to control the molecular size of γ-PGA by making good use of this characteristic of Bacillus sp. SJ-10. This result is worth because so far the 400 kDa γ-PGA produced by microorganism is rare.

3.4. Optimization of fermentation conditions for production of ~400 kDa γ-PGA

3.4.1. Temperature and pH

In order to evaluate the effects of temperature and pH on the γ-PGA production, fermentations were carried out at 4-50℃ and initial pH 5-11. Additionally, the relative γ-PGA yield in fermentation media have been determined at various concentrations to estimate mutual interaction

affecting the production of γ-PGA and growth between the L-glutamic acid and NaCl as precursor and inducer, respectively. First of all, the fermentation on temperature effects was done at pH 7.5 for 72 h shaking at 150 rpm with glucose and NH_4Cl as carbon and nitrogen source, respectively. Results are shown in Fig. 6. The optimum temperature for both γ-PGA yield and cell growth was found to be 37℃ (Fig. 6A). When relative productivity was assumed to be 100% at 37℃, the production yield was shown over 60% relative productivity in the temperature range at 25-45℃. The production yield was 64.8, 82.2 and 61.5% at 25, 30 and 45℃, respectively. Whereas, the SJ-10 showed low γ-PGA production and cell growth at obviously low or high temperatures, 4, 10 and 55℃. Although the pH range of 6 to 8 gave higher cell growth compared with other initial pH controlled fermentation media at 37℃, the higher γ-PGA production was recorded with pH ranging from 7 to 8 (Fig. 6B).

3.4.2. L-glutamic acid

The effect of L-glutamic acid on γ-PGA production was investigated in the range of 0–50 g/L with glucose and NH_4Cl as carbon and nitrogen source, respectively, at 37℃ in pH 7, as shown in Fig. 6C. Although *Bacillus* sp. SJ-10 produced γ-PGA in the absence of additional glutamic acid, γ-PGA yield was increased in the concentration dependent manner of added glutamic acid in the range of 0-30 g/L L-glutamic acid. The γ-PGA was accumulated to be almost constant yield at the 30g/L more levels of exogenous glutamic acid.

γ-PGA producing bacteria have been divided into two groups depending upon their nutrient requirement. One, glutamic acid independent producers, does not require L-glutamic acid in the medium. The other, glutamic acid-dependent producers such as *Bacillus* sp. SJ-10, requires the addition of L-glutamic acid for enhanced productivity. However, glutamic acid-dependent producers can synthesize considerable γ-PGA even in the absence of exogenously supplied L-glutamic acid due to the resource of L-glutamic acid obtained through the de novo pathway [33]. The L-glutamate-dependent producers are more desirable for industrial γ-PGA production due to

higher γ-PGA productivity compared with those of L-glutamate independent producers despite their low cost of production and simple fermentation process.

3.4.3. NaCl concentrations

The effect of NaCl on γ-PGA yield was shown in Fig. 6D. Although the cell growth was same level at the end of 3days fermentation up to 10% NaCl concentration, the γ-PGA yield was enhanced when the NaCl concentrations increased up to 6% with highest γ-PGA yield. In the presence of 6% NaCl, its yield was increased by 4 times compared with that of the without additional NaCl. In the presence of 8% NaCl concentration that produced constant molecular weight γ-PGA, ~400kDa, its relative productivity was obtained about 95% yield compared with presence of 6% NaCl.

To the best our knowledge so far, two halotolerant *Bacillus* which be affected by salt in γ-PGA production have been reported, suggesting that NaCl had a positive effect on the γ-PGA production such as *Bacillus* sp. SJ-10. Although *B. megaterium* never accumulated the exopolymer in salt-poor liquid media (0-0.5% of NaCl), increasing the salt concentration, more than 2% NaCl, resulted in a dramatic increase in the polymer yield that the maximum volumetric yield of γ-PGA from liquid cultures is 8.6 mg/mL in 5% NaCl [34]. The *B. licheniformis* WX-02 also showed that the highest yield of 13.86 g/L was obtained when the NaCl concentration was 8%, increasing by 5.28 times as compared with that of the control without NaCl. The γ-PGA productivity was enhanced as the NaCl concentration increased, which suggested that the γ-PGA was synthesized to increase the survival of *B. licheniformis* WX-02 when exposed to high osmotic stress by hypersalinity condition. γ-PGA might support the hydration status of *B. licheniformis* under strong dehydrating conditions based on its strong water-binding capacity [19]. Therefore, the γ-PGA produced by SJ-10 with maximum yield in the presence of 4-8% NaCl might also possessed *B. licheniformis* WX-02-like functionality.

According to regulators related to γ-PGA production in *B. subtilis* reported by Stanley and

Lazazzera [35], the DegS-DegU regulator proteins activated the transcription of *degQ*, which activated the transcription of the *ywsC* operon responsible for activating γ-PGA biosynthesis. Additionally, these regulators were suggested that are affected by osmolarity, high cell density and phase variation. Furthermore, according to Ruzal and Sanchez-Rivas [36], hypertonic media by high salinity could activate the transcription of DegSU in *B. subtilis* QB4256. Consequently, it was possible that NaCl activated transcription of the *ywsC* operon finally promoted the γ-PGA production. From previous results, it is suggested that these processes could be the main reason of the salt-inducible γ-PGA production from *Bacillus* sp. SJ-10.

3.5. The productivity of 400 kDa γ-PGA in accordance with carbon and nitrogen sources under the optimized condition

The effect of various C- and N- source on productivity of 400 kDa γ-PGA under the optimized culture condition was investigated. The glucose and ammonium chloride in the fermentation medium was replaced by other C- source (glucose, sucrose, starch, galactose, glycerol, maltose, mannose, xylose, manitol, lactose and fructose) and N- source (NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, NaNO3, yeast extract, L-alginine and peptone) as the final concentration of 3% and 1%, respectably. The fermentation was done 3% L-glutamic acid and 8% NaCl in 37°C at pH 7 for 3 days with shaking at 150 rpm. As shown in table 1, sucrose and NH_4Cl were favorable for γ-PGA production. The 24.7 g/L of γ-PGA was produced in the medium after 3 days fermentation. In the case of starch with NH_4Cl, also a high yield of γ-PGA was attained as 24.0 g/L compared with other nutrients sources. Regardless of molecular weight, the maximum production yield of γ-PGA by *Bacillus* sp. SJ-10 was 26.2 g/L under above optimized culture condition with sucrose and NH_4Cl as carbon and nitrogen sources, respectively, in 6% NaCl concentration.

3.6. Total antioxidant activity

The total antioxidant activity of γ-PGA was measured using ABTS radical scavenging and phosphomolybdenum assay. Both antioxidant activity determined by the two methods was

increased in a concentration dependent manner (Fig. 7). The ABTS radical scavenging activity is based on the decolorization of ABTS radicals, that is, radical cation ABTS+ was reduced to ABTS−. The Fig. 7A was shown that γ-PGA scavenges ABTS radicals with maximal reduction at 0.5 mg/mL of γ-PGA. Quantitative antioxidant determination by phosphomolybdenum method is based on the reduction of molybdenum (Mo VI to Mo V) by the sample. As shown in Fig. 7B, the 1 mg/mL γ-PGA displayed the maximum activity with 20 μg ascorbic acid equivalent.

According to our survey, there was just one report to describe the antioxidant activity of γ-PGA produced by *B. subtilis* D7 isolated from Korean fermented soy paste [37]. A γ-PGA from *B. subtilis* D7 scavenged the ABTS radical in a concentration dependent manner, with maximal reduction of 42.1 ± 1.2% at 10.0 mg/mL of γ-PGA. The 400 kDa γ-PGA from *Bacillus* sp. SJ-10 exhibits 2.4 times higher maximum activity with even the 10 times less amount compared with γ-PGA produced by *B. subtilis* D7. In view of this, γ-PGA is a potential alternative to new dietary supplement and functional food. Accordingly, the antioxidant properties of γ-PGA could ultimately lead to application in foods, cosmetics, and pharmaceuticals for the control of oxidative damage caused by reactive oxygen species. We are currently performing studies to further elucidate the antioxidant activity and its mechanism.

4. Conclusion

Physicochemical properties of sodium salt form γ-PGA produced by *Bacillus* sp. SJ-10 have been characterized in present work by FTIR, NMR, TGA and TLC analysis. Advantageous characteristics of the strain SJ-10 as a γ-PGA producer were found that sodium salt is a significant factor of molecular weight regulation and its production. The molecular weight was become constant to ~400 kDa when the increased NaCl concentrations more than 8%, and 400 kDa γ-PGA exhibits strong total antioxidant activity. These results suggest that availability in the food, feed supplement and biomedical industry such as cosmetic for the products development of certain quality with antioxidant activity as well as provide an economical method for controlling the constant molecular weight.

Table and Figure

Table 1. Effect of various carbon and nitrogen sources on productivity (g/L) of 400 kDa γ-PGA under the optimized condition[a]

C-source (30g/L)	N-source (10g/L)						
	NH_4Cl	$(NH_4)_2SO_4$	NH_4NO_3	$NaNO_3$	Yeast extract	L-alginine	Peptone
Glucose	21.7 ± 1.0	21.1 ± 1.2	17.0 ± 1.1	13.9 ± 0.8	22.1 ± 1.1	6.6 ± 0.3	2.7 ± 0.1
Sucrose	24.7 ± 1.1	22.7 ± 1.1	18.5 ± 1.1	16.4 ± 0.7	25.9 ± 1.3	7.7 ± 0.4	3.7 ± 0.2
Starch	24.0 ± 1.1	21.5 ± 1.1	17.9 ± 1.1	16.1 ± 0.8	24.4 ± 1.3	6.3 ± 0.3	3.3 ± 0.2
Galactose	20.0 ± 1.0	17.6 ± 1.0	14.3 ± 0.9	12.8 ± 0.6	19.4 ± 1.1	5.8 ± 0.1	2.8 ± 0.1
Glycerol	18.8 ± 1.1	16.6 ± 0.9	11.7 ± 0.8	11.5 ± 0.6	20.4 ± 1.0	5.5 ± 0.2	2.5 ± 0.1
Maltose	17.0 ± 0.5	16.6 ± 0.9	14.8 ± 0.9	11.6 ± 0.6	19.5 ± 0.7	4.6 ± 0.1	2.3 ± 0.1
Mannose	16.6 ± 0.8	16.4 ± 0.8	14.3 ± 0.8	11.5 ± 0.4	17.8 ± 0.9	4.1 ± 0.1	2.3 ± 0.1
Xylose	16.3 ± 0.8	13.7 ± 0.8	13.0 ± 0.7	10.4 ± 0.6	15.0 ± 0.8	5.0 ± 0.1	2.1 ± 0.1
Manitol	15.9 ± 0.9	14.6 ± 0.8	12.0 ± 0.7	10.2 ± 0.5	17.2 ± 0.9	4.2 ± 0.2	2.0 ± 0.1
Lactose	11.9 ± 0.6	11.0 ± 0.5	8.8 ± 0.5	7.0 ± 0.3	14.4 ± 0.7	3.0 ± 0.1	0.8 ± 0.1
Fructose	9.3 ± 0.3	8.5 ± 0.4	6. ± 0.6	5.9 ± 0.3	10.3 ± 0.6	1.4 ± 0.2	-

[a]The data in this study were derived from experiments repeated at least three times and the experimental errors were less than 5%

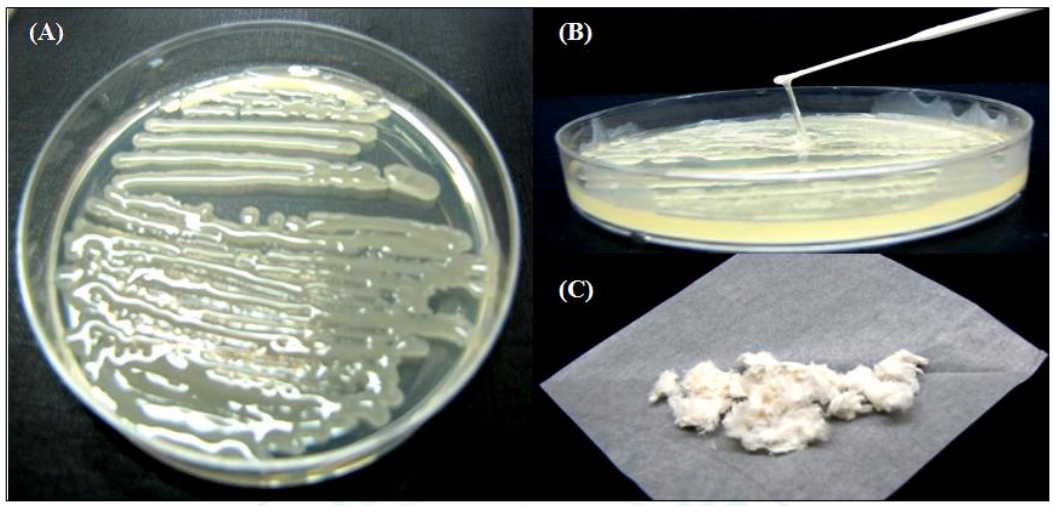

Fig. 1. *Bacillus* sp. SJ-10 in solid media and purified γ-PGA powder. (A); The SJ-10 on HM plate at 37℃ after 24 h. (B); Slimy characteristic of the SJ-10. (C); Purified γ-PGA powder.

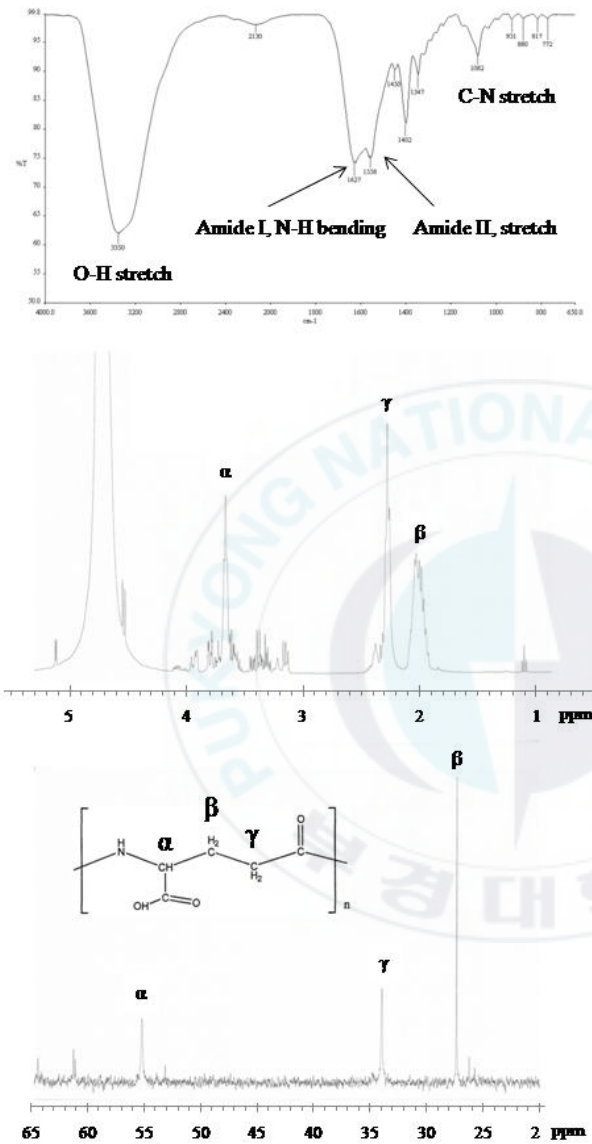

Fig. 2. The chemical structure of biopolymer isolated from SJ-10. FT-IR absorption spectrum (A), ^{1}H-NMR (B) and ^{13}C-NMR (C).

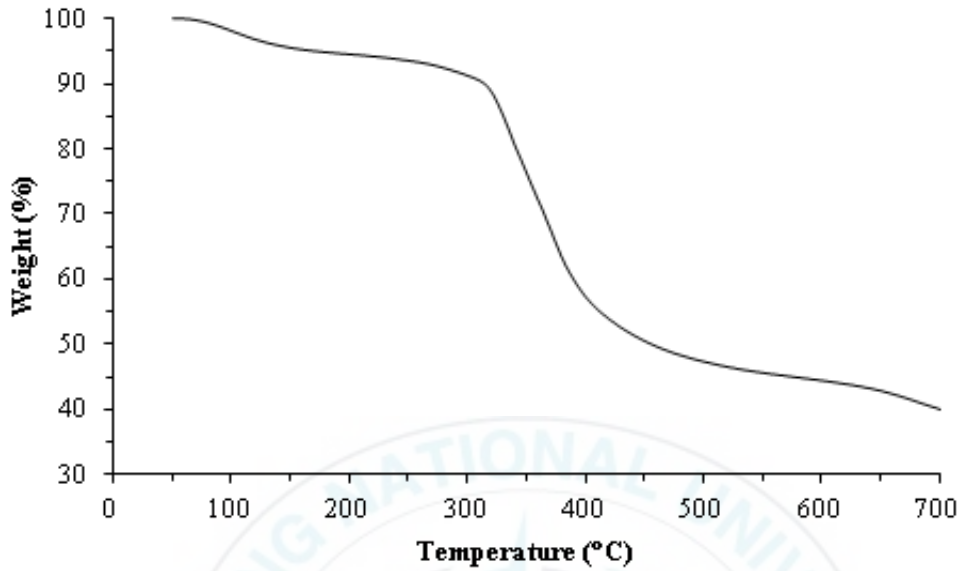

Fig. 3. Thermal gravimetric analysis of purified γ-PGA.

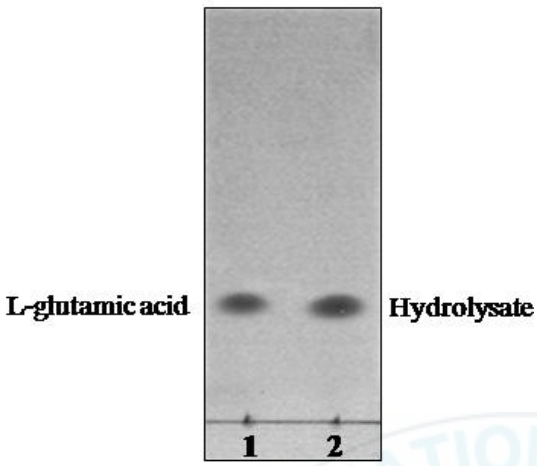

Fig. 4. Thin layer chromatography analysis of biopolymer produced by *Bacillus* sp. SJ-10. Lane 1, L-glutamic acid solution; lanes 2, hydrolysates of γ-PGA isolated from *Bacillus* sp. SJ-10.

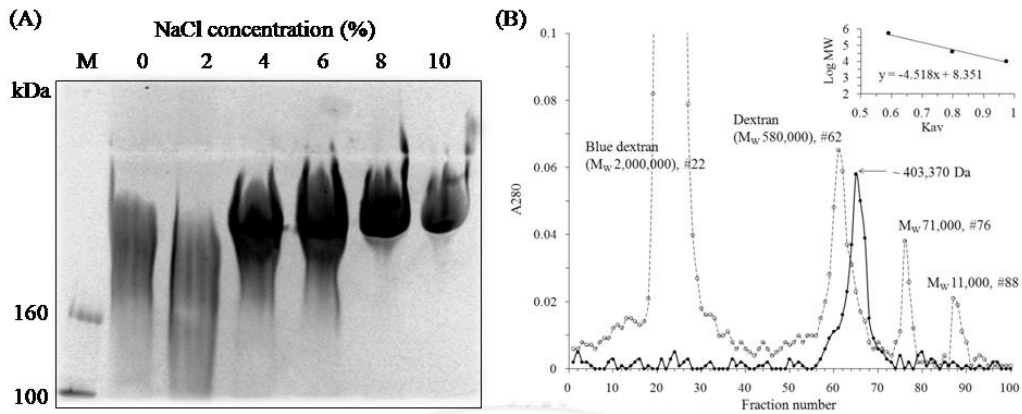

Fig. 5. NaCl effect on the molecular weight of extracellular biopolymer. (A); SDS-PAGE profiles of extracellular biopolymer obtained at different NaCl concentration of cultivation. Lane M; molecular weight markers, Lane 0-10 indicate the NaCl concentration (%), (B); Molecular weight determination of the polymer produced in 8% NaCl by the gel filtration from a Sephadex column with dextrans standards.

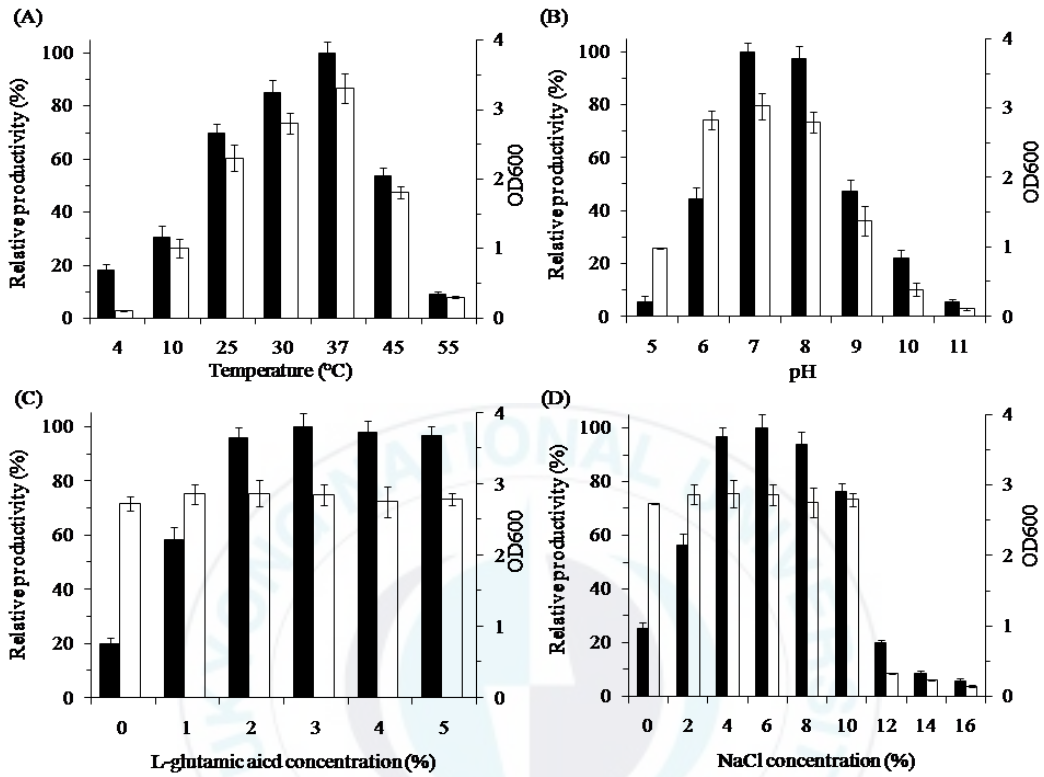

Fig. 6. Relative productivity of γ-PGA in accordance with the culture conditions. (A); temperature, (B); pH, (C); L-glutamic acid, (D); NaCl. ■; Relative productivity, □; cell growth at OD_{600}

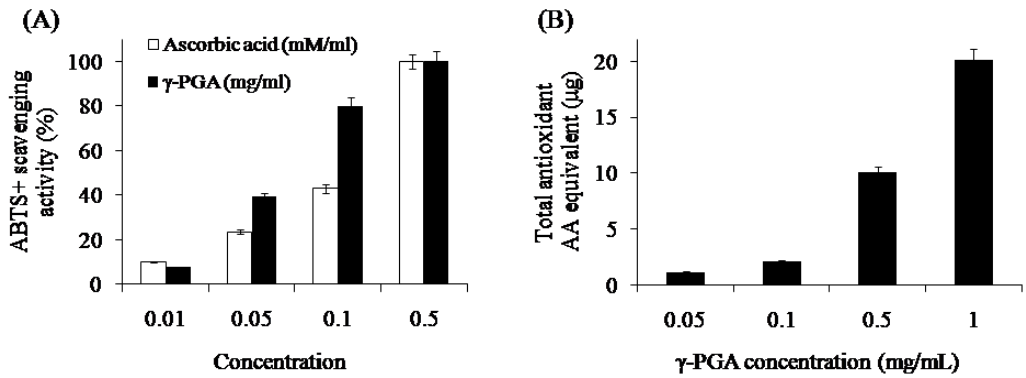

Fig. 7. Total antioxidant activity of γ-PGA using ABTS+ scavenging (A) and phosphor-molybdenum assay (B).

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Chapter 3

Antioxidant mechanism and gastrointestinal cytoprotective effect of γ-PGA against hydroxyl radical induced damage

Abstract

Antioxidant mechanism and cytoprotection of the γ-PGA produced by halotolerant *Bacillus* sp. SJ-10 were investigated. The γ-PGA with molecular weight ~400 kDa showed significantly hydroxyl radical averting capacity as 85.2% at 1mg/mL, and it was caused by chelating transition metal ions and scavenging hydrogen peroxide. There was no significant variation in hydroxyl radical averting capacity of γ-PGA after 9 h, end point of simulated digestion, by retaining activity of 95.3% compared to untreated γ-PGA. Furthermore, the γ-PGA showed protective effect against DNA, protein and Caco-2 cell damage by oxidative damage induced hydroxyl radical, in particular it show completely protective effect at 1mg/ml. These data suggest that γ-PGA produced by SJ-10 has a potential use as a cytoprotectant in the food and feed supplements, cosmetics and biomedical fields.

Keywords; γ-PGA, Antioxidant, Hydroxyl radical, Cytoprotection

1. Introduction

Reactive oxygen species (ROS) caused by different ways contribute to the oxidative stress in living organisms. When produced in excess ROS can cause tissue injury and reduction of protein function by attacking cellular components and amino acids. In addition, the overproduction of ROS have been implicated in most of diseases, including aging, arthritis, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, and cancer [1, 2]. All aerobic organisms, including human beings, have defense systems to protect biomolecules from oxidative damage. However, these own natural antioxidant defence system cannot remove all generated ROS. Therefore, dietary intake of antioxidant compounds becomes important in order to prevent oxidative stress, and there is continuous demand for exogenous natural antioxidants. Although there are some synthetic antioxidant compounds, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) which are commonly used in processed foods, it has been reported that these compounds have some side effects [3, 4]. Therefore, with safety concerns identified for these synthetic antioxidants, considerable interest has arisen on the study for the natural antioxidants source.

Naturally occurring polymers have attracted considerable interest in recent years because, in view of sustainability, recyclability, and environmental responsibility, they offer a possible alternative to synthetic substances [5]. Therefore, a cheap and easy alternative, which is currently gaining popularity, is microbes. A poly-γ-glutamic acid (γ-PGA), as microbial biopolymer, ideally meets the demands of the times that preservation of environmental systems as well as well-being issues for a healthy life [6]. γ-PGA is an unusual anionic, water-soluble, edible and naturally occurring homo-polyamide that is made of D- and L-glutamic acid units connected by amide linkages between α–amino and γ–carboxylic acid groups. It can either be composed of only L-glutamic acid residues (γ-L-PGA), only D-glutamic acid residues (γ-D-PGA) or both L- and D-glutamic acid residues (γ-LD-PGA) [7]. γ-PGA possesses multi-functionalities, its application –

biodegradable substitutes (as thermoplastics, fibers, films and membranes), hydrogels with very high water-absorption capability, flocculants, heavy metal- and radionuclide binding agents, cryoprotectants, bitterness-relieving agents, thickeners, animal feed additives, osteoporosis-preventing factors, humectants, drug deliverers, gene vectors, curative biological adhesives, dispersants, and enzyme-immobilizing materials, for example – now attracts particular attention [8]. The γ-PGA produced mostly by Gram-positive bacteria which includes the *Bacillus* genus can exhibit different properties according to conformational states, enantiomeric properties and molecular weight. In addition, it can exist either in the water insoluble free acid form or as its salt with a variety of cations (Na^+, Mg^{2+}, K^+, NH^{4+} or Ca^{2+}) which is completely soluble [9]. Therefore, γ-PGA has diverse biochemical properties, enabling it to play different roles, depending on the organism and its environment. Indeed, γ-PGA allows bacteria to survive at high salt concentrations and may also be involved in adhesion.

Halotolerant microorganisms able to live in high salinity environments, offer a multitude of actual or potential applications in various fields of biotechnology. In comparison to normal environment, unique habitat with hyper variable conditions could represent the novel functional abilities of the microbes that can be further elucidated for their potential as source of natural products. Commonly, halophilic or halotolerant bacteria are characterized by a much greater metabolic diversity and useful macromolecular substances are active and stable at high salt contents [10]. In the previous study, a novel *Bacillus* spp, *Bacillus* sp. SJ-10, was isolated from *jeotgal*, a traditional Korean salted and fermented seafood, and it was confirmed the γ-PGA having relatively constant molecular weight from high salinity batch fermentation. In this work, antioxidant activity and its mechanism of γ-PGA, and protective effects against hydroxyl radical induced damage were investigated by a series of experiments.

2. Materials and methods

2.1. γ-PGA preparation

The γ-PGA production strain, *Bacillus* sp. SJ-10 (KCCM 90078, JCM 15709), was originally isolated from *jeotgal*, the Korean salted and fermented seafood, in our previous study [11]. The strain was grown in a γ-PGA production medium composed of glucose (10 g/L), NH_4Cl (5 g/L), Sodium chloride (80 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $CaCl_2 \cdot 2H_2O$ (0.15 g/L), $MnSO_4 \cdot 4H_2O$ (0.15 g/L) and $FeCl_3 \cdot 7H_2O$ (0.04 g/L). After fermentation for 5 days, the viscous culture broth was separated to remove the cells and solid particles by centrifugation for 10 min at 6,000 rpm. The supernatant was collected, and mixed with three volumes of absolute ice-cold ethanol. The mixture was stored at 4°C for 12 h to precipitate γ-PGA, which was recovered by centrifugation at 12,000 rpm for 20 min. After centrifugation, the resultant precipitate was collected, dissolved in appropriate volume of distilled water, boiling for 10min and then centrifuged to remove any insoluble contaminants. The aqueous γ-PGA was desalted by dialysis in distilled water at 4°C overnight. Finally, the lyophilized γ-PGA was used for further analyses.

2.2. Hydroxyl radical averting capacity

The hydroxyl radical averting capacity was evaluated with slight modifications on the Fenton reaction described by Yuan et al., 2012 [12]. Firstly, 100 µL sample was mixed with 50 µL ferrous sulphate (7.5 mM), 50 µL 1,10-phenanthroline (7.5 mM) and 750 µL phosphate buffer (100 mM, pH 7.4). Then 50 µL of 0.1% hydrogen peroxide was added to the mixture to initiate the reaction. After incubation for 30 min at 37°C, the absorbance of the mixture was measured at 560 nm using a UV/Vis spectrophotometer (OPTIZEN POP, Mecasys, Korea). The ascorbic acid, BHT and BHA were used as reference. Hydroxyl radical averting capacity was expressed by the following equation; Averting capacity (%) = [(As-A1)/(Ao-A1)] × 100

where Ao is the absorbance of control without test sample and H_2O_2, A1 is the absorbance of control without test sample and As is the absorbance of the test sample.

2.3. Non site-specific and site-specific hydroxyl radical-mediated 2-deoxy-D-ribose degradation

The ability of the extracts to inhibit non site-specific hydroxyl radical-mediated peroxidation was carried out according to Hinneburg et al., 2006, with some modification [13]. The reaction mixture contained 200 µL of γ-PGA dissolved in phosphate buffer (100 mM, pH 7.4), 200 µL of 30 mM 2-deoxy-D-ribose in phosphate buffer, 200 µL of premixed 1 mM $FeCl_3$ and 1.4 mM EDTA (1:1 v/v) solution, 200 µL of 20 mM H_2O_2 and 100 µL of 2 mM aqueous ascorbic acid was vortexed and incubated at 37℃ for 30 min. Thereafter, 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were added to each tube, and the samples were vortexed and boiling for 10 min. The extent of oxidation was estimated from the absorbance of the solution at 532 nm. The ability of the γ-PGA to inhibit site-specific hydroxyl radical-mediated peroxidation was carried out as described in the non site-specific hydroxyl radical mediated peroxidation inhibition procedure, except that EDTA was replaced by buffer. The percentage of inhibition values were calculated by the following equation; Inhibition (%) = [(Ac–As)/Ac] × 100

where Ac is the absorbance of control without test sample, As is the absorbance of the test sample.

2.4. Iron ions chelating activity

The ferrous and ferric ions chelating by γ-PGA and standards were estimated by the ferrozine method [14]. Briefly, the 100 µL (0 ~ 1 mg/mL) sample was added to a solution of 2mM $FeCl_3$ or $FeSO_4$ (900 µL). The mixture was shaken vigorously and left standing at room temperature for 10 min. And then the mixture was centrifuged for 10 min at 12000 rpm. The 100 µL supernatant was added 15 µL reducing agent (1.4 M hydroxylamine in 2M hydrochloric acid) and left standing at room temperature for 10 min. NEXT, the mixture was reacted with a solution composed of 750 µL deionized water, 100 µL ferrozine (5mM) and 5M ammonium acetate (pH 9.5 with a solution of ammonium hydroxide). After left standing at room temperature for 10 min, the absorbance of the reaction solution was measured spectrophotometrically at 562 nm. The chelating activity of γ-

PGA against Fe^{2+} was carried out as described in the Fe^{3+} chelating activity procedure, except that reducing agent was replaced by deionized water. The inhibition rate of ferrozine-Fe^{2+} complex formation was given bellow formula; Iron ions chelating activity (%) = [(A0-A1)/A0] × 100

where A0 was the absorbance of the control and A1 was the absorbance in the presence of the sample. The ferrozine-Fe^{2+} complex solution was used as control.

2.5. Fourier transform infrared (FT-IR) spectra

The freeze-dried purified γ-PGA and Fe^{3+}-γ-PGA sample were used for FT-IR measurements in the frequency range of 650–4000 cm^{-1} using Perkin Elmer (USA), Spectrum X. The samples were placed at the surface of the ATR crystal, and a pressure was applied to expel air from the powder particles. The spectrum of the sample was collected by averaging 128 scans at four resolutions.

2.6. Hydrogen peroxide scavenging activity

The ability of the γ-PGA to scavenge hydrogen peroxide was determined according to the method of Ruch et al., 1989 [15]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined by spectrophotometer at 230 nm. γ-PGA in 100 µL distilled water with various concentrations were added to a hydrogen peroxide solution (900 µL). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing in phosphate buffer without hydrogen peroxide. The catalase from bovine liver (Sigma) was used as reference. The hydrogen peroxide scavenging rate of γ-PGA and standard compounds was calculated by fallowing equation; H_2O_2 scavenging activity (%) = [(A0-A1)/A0] × 100

where A0 was the absorbance of the control, and A1 was the absorbance in the presence of γ-PGA.

2.7. In vitro digestion model

In vitro digestion model was used according a method reported previously with slight modifications [16]. Initially, to simulate saliva condition, purified γ-PGA (0.2 g) was transferred to clean amber bottles and mixed with artificial saliva solution (12.0 g/L NaCl, 14.4 g/ml KCl, 4.4

g/mL $CaCl_2$, 30.0 g/mL $NaHCO_3$) to create a final volume of 20 ml. And then the sample was incubated at 37 °C in a shaking water bath at 95 rpm for 1 h. Next, the samples were acidified to pH 2 with 1 mL of porcine pepsin preparation (0.04 g pepsinin 0.1 mol/L HCl) and incubated at 37 °C in a shaking water bath at 95 rpm for 3 h. After gastric digestion, the pH was increased to 5.3 with 0.9 mol/L sodium bicarbonate, and then 200 µL of the bile salts glycodeoxycholate (0.04 g in 1 mL of saline), taurodeoxy-cholate (0.025 g in 1 mL of saline), taurocholate (0.04 g in 1 mL of saline), and 100 µL of pancreatin (0.04 g in 500 mL saline) were added. The pH of each sample was increased to pH 7.4 with 1 mol/L NaOH and the samples were incubated at 37 °C in a shaking water bath at 95 rpm for 5 h to complete the intestinal phase of the in vitro digestion process. After the intestinal digestion phase, 1 mL of each sample were corrected and stored at -20 °C. Samples were analyzed within 2 weeks.

2.8. Protective effect on Hydroxyl radical mediated degradation of DNA and protein

Hydroxyl radical mediated oxidation of DNA (λ DNA) and protein (bovine serum albumin; BSA) were carried out by metal-catalyzed reaction based on Galano et al., 2010, and Kocha et al., 1997, with some modifications [17, 18]. Briefly, 38.5 µL of solutions of γ-PGA (0-1 mg/mL) dissolved in a solution of ascorbic acid (0.8 mM)/EDTA (0.4 mM)/$FeSO_4$ (0.4 mM) were mixed with 10 µL of DNA solution (1 µg/µL) and 1.5 µL of 2% H_2O_2. In the control tube (without the hydroxyl radical generator system), the H_2O_2 was replaced by deionized water. After incubation for 3h at room temperature, the reaction was stopped by addition of 50 µL loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 15% ficoll 400). A 10 µL of this mixture was electrophoresed in 1% agarose gel containing ethidium bromide (0.5 µg/mL).

Experiments for hydroxyl radical mediated oxidation of protein were performed similarly to DNA oxidation assays. A ascorbic acid (0.8 mM)/EDTA (0.4 mM)/ $FeSO_4$ (0.4 mM) solution was prepared in 50 mM phosphate buffer, pH 7.4, and BSA and γ-PGA (0-1 mg) were dissolved in it. The assay was made as follows: A 1% BSA (250 µL) and ascorbic acid/EDTA/$FeSO_4$ solution

(250 μL) was mixed in 1.5 ml eppendorf tube with or without γ-PGA. The generation of hydroxyl radical was initiated with the addition of 15 μL of 2% H_2O_2. In the control tube (without the hydroxyl radical generator system), the H_2O_2 was replaced by water. After 3h of incubation at room temperature, 250 μL of 20% trichloroacetic acid was added, and the mixture was centrifuged at 2000g for 30 min at 4℃. The supernatant was discarded, and the pellet was resuspended with 500 μL of 0.1 M NaOH. To evaluate the protein damage induced by hydroxyl radical, the 10 μL sample was subjected to SDS-PAGE. After running in 12.5% polyacrylamide gel, the gels were stained with 0.2% coomassie brilliant blue R for 1 h and destained with deionized water.

2.9. Cytotoxicity of γ-PGA on CaCo-2 cell

Caco-2 cells (KCLB 30037) were obtained from the Korean Cell Line Bank (KCLB). The cells were grown at 37℃ in an atmosphere of 95% air and 5% CO_2 in minimal essential medium (MEM, Sigma M0643) supplemented with 25 mM HEPES, 10% heat-inactivated fetal bovine serum (FBS, Gibco 16000), 1% non-essential amino acids and 1% penicillin/streptomycin (Gibco, Grand Island, NY). The cytotoxic effect of γ-PGA was conducted by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Caco-2 cells (5×10^4) were seeded and cultured in 96-well cell plates with maintenance medium at 37℃. After 24 h, the culture medium was changed with 100 μL fresh medium supplied with various concentrations of γ-PGA (0.25, 0.5 and 1.0 mg/mL), and the cells were stimulated for 48 h. Then, 20 μL MTS solution was added to each well and cultured for 1 h. The absorbance was evaluated at 570 nm using a multidetection microplate reader.

2.10. Cytoprotection of γ-PGA against hydroxyl radical

The cytoprotective effect against hydroxyl radical damage was done by co-incubation method. Caco-2 cells were cultured in 48-well plates for 72 h before hydroxyl radical oxidative stress induction. Prior to experiment, culture media of caco-2 cells were removed and cells were washed

twice with PBS. A 500 μL PBS which dissolved appropriate concentration of the γ-PGA was added in cultured well plate, and then same volume of hydroxyl radical generator solution was added. The hydroxyl radical generate system was prepared that solution was composed of ascorbic acid (0.8 mM)/EDTA (0.4 mM)/ FeSO4 (0.4 mM) in 500 μL PBS. The generation of hydroxyl radical was initiated with the addition of 17 μL of 2% H_2O_2. In the control tube, the hydroxyl radical generator solution and H_2O_2 were replaced by PBS. After 3 h incubation at 37 ℃ in 5% CO_2 incubator, cell viability was measured by MTS assay as described above.

3. Results and discussion

3.1. Hydroxyl radical averting capacity of γ-PGA

Hydroxyl radical averting capacity of γ-PGA was investigated with ascorbic acid, BHT and BHA as references and shown in Fig. 1. The γ-PGA exhibited averting capacity on hydroxyl radical in a concentration-dependent manner. The averting capacity was exponentially increase up to 0.1 mg/mL of γ-PGA, and after then the increasing rate was shown to decrease gradually up to 1 mg/ml. In this assay, the estimated IC_{50} of γ-PGA, ascorbic acid and BHT were 131, 89 and 65 μg/mL, respectively, however, at 1 mg/mL, γ-PGA showed an excellent hydroxyl radical averting capacity (85.2%) more other standard compounds, ascorbic acid (80.1%), BHT (62.4%) and BHA (28.5%).

In hydroxyl radical averting assay, there are two types of antioxidant mechanism; which are suppression of hydroxyl radical generation and directly elimination of the generated hydroxyl radical. The former mechanism, suppression of hydroxyl radical, was related to the transition metal ions and hydrogen peroxide. In the absence of transition metal ions, hydrogen peroxide is quite stable. However, hydroxyl radicals acted in superoxidation by hydrogen peroxide with metal ions, usually ferrous or cupper. The molecules which could chelate irons and convert hydrogen peroxide in Fenton reaction might have scavenging ability on hydroxyl radical [19]. Therefore, we have examined how the γ-PGA will block hydroxyl radical.

3.2. Non site-specific and site specific scavenging activity

The both non-site specific and site specific hydroxyl radical scavenging activity of γ-PGA were increased in a concentration-dependent manner (Fig. 2). Additionally, considerable difference between the performance in the site-specific and the non site-specific assays were found. The γ-PGA performed better in the site-specific assay. In the site-specific assay, hydroxyl radical scavenging activities were 35.2, 51.9, 67.9 and 80.3% at 0.1, 0.25, 0.5 and 1.0 mg/mL, respectively. Meanwhile, scavenging activity in non site-specific assay was exhibited 8.1, 18.3,

26.4 and 35.2% at 0.1, 0.25, 0.5 and 1.0 mg/mL, respectively. Overall, γ-PGA showed 2 times more high activity in site-specific assay than non-site-specific assay.

The results from the inhibition of deoxyribose degradation might reveal some of the hydroxyl radical averting capacity mechanisms of γ-PGA. In the non site-specific assay, EDTA forms a complex with Fe^{3+}, and hydroxyl radicals are generated in solution containing ascorbic acid and hydrogen peroxide. Meanwhile, in the site-specific assay, EDTA is omitted. Therefore, Fe^{3+} can bind to deoxyribose and produce hydroxyl radicals at this site. So iron chelating compounds can reduce the extent of deoxyribose degradation even if they are not effective hydroxyl radical-scavengers. In the non site-specific assay, this only influences the results if the compounds directly remove the hydroxyl radical and/or form a more stable complex with Fe^{3+} than EDTA. The γ-PGA which performed better in the site-specific than in the non site-specific assay was supposed to be better iron chelators than hydroxyl radical scavengers. Thus γ-PGA was thought to be higher iron chelating ability than directly scavenging activity for hydroxyl radicals. For this reason, we have also checked iron chelating activity of the γ-PGA.

3.3. Iron chelating ability

The chelation of iron ions by γ-PGA was estimated by detecting color change using ferrozine in which quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the formation of Fe^{2+}-ferrozine complex are disrupted, thereby the red color change is impeded. Measurement of this color change therefore allows for the estimation of the chelating activity of the coexisting chelator. As shown in Fig. 3, the formation of the Fe^{2+}-ferrozine complex was not complete in the presence of the γ-PGA in both Fe^{3+} and Fe^{2+} solution, indicating that γ-PGA chelate the iron. The iron ion chelating effect of γ-PGA was related to its concentrations, and the chelation on Fe^{3+} ion was slightly higher than on Fe^{2+}. The formation of Fe^{2+}-ferrozine complex linearly decreased in a γ-PGA dose-dependent manner from 0.1 mg/mL and 0.25 mg/mL against Fe^{3+} and Fe^{2+}, moreover, complete chelating capacities of γ-PGA concentrations were 0.5 and 1 mg/mL, on Fe^{3+} and Fe^{2+},

respectively. The γ-PGA showed excellent chelating ability against both ferric and ferrous ions more than other tested antioxidant compounds.

Among the transition metals, iron is known as the most powerful prooxidant due to its high reactivity. Iron generate hydroxyl radical and stimulate lipid peroxidation by the Fenton reaction, result in induced degradation of biological macromolecules and accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. Iron chelator can afford to protection against metal-catalyzed oxidative damage by inhibiting production of hydroxyl radical and lipid peroxidation in Fenton type reactions. Therefore, the iron chelating capacity is significant since it reduces the concentration of the catalyzing transition metal iron. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion [20]. The data obtained from Fig. 3 reveal that γ-PGA demonstrates a strong capacity for iron binding, suggesting protective effect on hydroxyl radical induced damage.

The analysis of FT-IR spectra of γ-PGA was done in our previous study (Fig. 2A in chapter 2). Briefly, the four major peaks were found at 1750 cm^{-1} representing carbonyl group: Amide I, N-H bending band at 1627 cm^{-1}; Amide II, stretching band at 1558 cm^{-1}; and C=O symmetric stretching band at 1402 cm^{-1}. Additionally, the characteristic peak observed at 2500 – 3600 cm^{-1} with broad band, overlap of O-H, N-H and C-H stretching vibrations. These appearance depicted typical peaks of γ-PGA. Moreover, it was revealed that specific two adjacent peaks, at about 1620 – 1655 cm^{-1} and 1550 – 1585 cm^{-1}, which is exhibited only pure sodium salt γ-PGA (Na^+ form) coincide with previous reports [21, 22]. The IR spectrum recorded for Fe(III)-γ-PGA complex showed a significant change at the broad band region around 1658.12 – 1537.44 cm^{-1}, implying the participation of amide and carboxyl groups in binding of mercury(II) on γ-PGA (data not shown).

3.4. Scavenging of hydrogen peroxide

The scavenging ability of γ-PGA on hydrogen peroxide was shown in Fig. 4, compared with catalase from bovine liver and antioxidants as reference compound. The γ-PGA was capable of scavenging hydrogen peroxide in an amount dependent manner. Among the reference antioxidants except catalase, ascorbic acid exhibited highest scavenging activity as 95.8% at concentration of 0.5 mg/mL, in the order of γ-PGA (92.4%) > BHT (88.1%) > BHA (45.6%) at 1mg/mL. This result showed that γ-PGA had strong hydrogen peroxide scavenging effect. On the other hand, The hydrogen peroxide scavenging activity values on IC_{50} which estimated concentrations in hydroxyl radical averting capacity assay of γ-PGA and standard compounds exhibited 80.1% on γ-PGA (131 μg/mL), 82.5% on ascorbic acid (89 μg/mL) and 80.1% on γ-PGA (131 μg/mL), respectively.

ROS are produced endogenously as normal products of cellular metabolism. One major contributor to oxidative damage is hydrogen peroxide, which is converted from superoxide that leaks from the mitochondria. Catalase and superoxide dismutase ameliorate the damaging effects of hydrogen peroxide and superoxide, respectively, by converting these compounds into oxygen and water, benign molecules. However, this conversion is not sufficiently effective enough to protect from oxidative stress, particularly when the ROS occurs excessive, and residual peroxides persist in the cell [23]. Furthermore, exogenous ROS can be produced from pollutants, tobacco, smoke, drugs, xenobiotics, or radiation. Ionizing radiation can generate damaging intermediates through the interaction with water, a process termed radiolysis. Since water comprises 55-60% of the human body, the probability of radiolysis is quite high under the presence of ionizing radiation. In the process, water loses an electron and become highly reactive. Then through a three-step chain reaction, water is sequentially converted to hydroxyl radical, hydrogen peroxide, superoxide radical and ultimately oxygen [24]. The hydroxyl radical is extremely reactive that immediately removes electrons from any molecule in its path, turning that molecule into a free radical and so

propagating a chain reaction. But hydrogen peroxide is actually more damaging to Biological system than hydroxyl radical since the lower reactivity of hydrogen peroxide provides enough time for the molecule to travel into the nucleus of the cell, subsequently wreaking havoc on macromolecules such as DNA, protein and lipid [25]. Thus, the removing of hydrogen peroxide is very important for antioxidant defense. In this sense, the γ-PGA can be a powerful antioxidant candidate.

3.5. Stability during in vitro simulated gastrointestinal tract

The hydroxyl radical averting capacity of the γ-PGA was significantly stable at a wide range of temperatures (10–100℃) and pH (pH 2–11) for 12 hr (Fig. 5). The relative Hydroxyl radical averting capacity of γ-PGA within that range was retained between 90% and 100%, even though activity levels were retained in 100℃ and at pH 2 and 11 as regarded extreme conditions.

Regarding hydroxyl radical averting capacity, it has been found that γ-PGA showed promising ability in vitro digestion model (Fig. 5). Concerning determination of the bioavailability of γ-PGA, we used an in vitro digestion model, which is designed to simulate *in vivo* digestion, because it gives an indication of the bioavailability of different biofunctional activities in a biological system. As shown in Fig. 5, we have found that the hydroxyl radical averting capacity tended to be lower during the simulated saliva stage than in the gastric and duodenal phases by time course *in vitro* simulated digestion model. However, the hydroxyl radical averting capacity of γ-PGA was gradually restored throughout the simulated gastric and duodenal phase. As a result, there was no significant variation in hydroxyl radical averting capacity of γ-PGA after 9 h, at end point of simulated digestion, by retaining activity of 95.3% compared to untreated γ-PGA. This variation in hydroxyl radical averting capacity after simulated saliva phase may have been due to light metal ions in the artificial saliva solution composed of $CaCl_2$, NaCl and KCl, because the interaction between light metal ions and γ-PGA to be predominantly electrostatic interaction. Nevertheless, the reason of maintenance of the high activity is that γ-PGA has a higher selectivity

for heavy metals, such as Fe^{3+} and Fe^{2+}, than Ca^{2+} [26, 27]. It is in agreement with previous reports by Inbaraj et al., 2009, that the light metal ions, being hard acids, can form ionic complexes with hard bases such as carboxylate anions in γ-PGA, and calcium ions showed a greater effect because of high positive charge [28]. Furthermore, in our FT-IR results suggest that ferrous ion was bind to carboxy group of γ-PGA. Fe ions chelation ability of γ-PGA in hydroxyl radical averting assay could be decreased due to competitive inhibition with light ions, especially $CaCl_2$ which present in saliva solution. Therefore, light ions may cause competitive inhibition in ions chelation ability of γ-PGA in artificial saliva solution. The previous report by Lee et al., 2006, found the effect of γ-PGA on the sustained release of Ca^{2+} in the small intestine [29]. The results of the present study strongly support these findings by gradually recovering the activity throughout simulated intestinal phase, particularly during duodenal phases. That is, after saliva phase, hydroxyl radical averting capacity of γ-PGA was gradually restored by the sustained release of Ca^{2+}, bound with γ-PGA including Na^{+} and K^{+}, in simulated small intestine phase. Furthermore, recovery of its activity is suggested that hydroxyl radical averting capacity of γ-PGA is not affected significantly the digestive enzymes and bile salt. Generally γ-PGA differs from proteins in terms of its structure. Its component glutamate residues are polymerized inside the cell via the γ-amide linkages and synthesized in a ribosome-independent manner [30]. Hence, γ-PGA is resistant to proteases which cleave α-amino linkages due to the γ-linkage of glutamate residues. Our data suggest that γ-PGA act as a bioavailable protector on hydroxyl radical and thereby prevented hydroxyl radical induced damage.

3.6. Protective effect of γ-PGA on DNA and protein

To confirm the DNA and protein protective effect of γ-PGA, hydroxyl radical-induced damage was conducted by hydroxyl radical generation system using $FeCl_3$, H_2O_2 and ascorbic acid with or without γ-PGA. After reaction, samples were run through 1% agarose gel electrophoresis on λ DNA and 12% SDS-PAGE on BSA, respectively. The Fig. 6 shows that γ-PGA is able to protect,

in a concentration-dependent way, λ DNA and BSA from the hydroxyl radical-induced degradation. The completely protection both λ DNA and BSA were observed with concentration of 1 mg/mL.

Hydroxyl radical, hydrogen peroxide and singlet oxygen together with one-electron process are involved in mutagenesis thereby induce a carcinogenesis and promote in aging. In this context, hydroxyl radical may be considered the main contributing reactive oxygen species to endogenous oxidation of cellular DNA [31]. The protection of DNA damage by γ-PGA is a consequence of their hydroxyl radical averting properties mainly chelating activity by removing transit metal ions from solution before they can complex with ascorbic acid. In addition to their averting capability, a hydrogen peroxide scavenging effect by γ-PGA cannot be ruled out and could also be important in the protection. Cellular protein structure is modified by alteration of amino acid side chain when proteins are damaged by reactive oxygen species. These modifications lead to disturbances of cellular metabolism by loss of their intrinsic function. Irreversible oxidation of amino acid residues in a protein can be exerted by two major mechanisms: ionizing radiation and metal ion-catalyzed oxidation reactions. The effects of both mechanisms are attributable to the hydroxyl radicals formed by the radiolysis of water or by catalyzing transition metal. The oxidative protein damages, provoked by hydroxyl radical, have been demonstrated to play a significant role in aging and several pathological events such as neurodegenerative diseases, diabetes and atherosclerosis [32]. Major molecular mechanisms, leading to structural changes in proteins are free-radical mediated protein oxidation characterized by carbonyl formation. Indeed, measurement of carbonyl formation has been used as a sensitive assay for oxidative damages of proteins [33]. The oxidation was determined in terms of carbonyl formation. γ-PGA dose-dependently exhibited the prevention on carbonyl formation and it showed almost completely inhibitory effect at the concentration of 1mg/ml (data not shown). These results suggest that the purified γ-PGA has the ability to protect DNA and protein from oxidative damage by hydroxyl radical, and it is a

reflection of its positive effects against many diseases in human body.

3.7. Cytotoxicity and cytoprotective effect to Caco-2 cell

The use of γ-PGA in food supplements and pharmaceuticals has been suggested especially as cryoprotectant, osteoporosis preventing agent, drug carrier/delivery and biological adhesive. These proposals are in line with the aim in this study which is intended for use as cytoprotectant on oxidative damage by hydroxyl radical. Therefore, its potential toxicity against human cell needs to be examined prior to investigate on cytoprotective effect. As shown in Fig. 7A, in vitro cytotoxicity analysis using MTS assay, we observed that Caco-2 cell viability did not show any inhibition and cytotoxic effect in all tested concentrations 0.01 ~ 1 mg/ mL of the γ-PGA. The non-cytotoxicity of γ-PGA produced by *Bacillus* sp. SJ-10 on caco-2 cell derived from human intestine is in agreement with the findings reported that no toxic effect on the human B-cell line EHRB and on mice has been evaluated [34]. In addition, non-toxic to γ-PGA has been reported despite extensive investigation of drugs that contain this polymer. In fact the traditional Asian foods made of soybeans fermented by *Bacillus* species, known as *Cheonggukjang* in Korea and *Natto* in Japan, show a typical slimy texture by high content of γ-PGA. It is a natural component found in the mucilage of traditional fermented soybeans, and it has a long history of safe use in food products.

To investigate the cytoprotective benefits, the effects of γ-PGA on H_2O_2–induced oxidative stress in Caco-2 cells were examined. H_2O_2 was used to induce ROS formation in this experiment because of its ability to pass freely across cell membranes. It is precursor of highly oxidizing, tissue-damaging radicals such hydroxyl radical as known to be highly toxic to many systems and generated from nearly all sources of oxidative stress. The exposure of Caco-2 cell to 1 mM H_2O_2 without γ-PGA for 3 h resulted in a significant reduction in cell viability as about 60.15% cell viability compared to the non-treatment group (Fig. 7B). The significant protective effect was not observed at the concentrations up to 0.1 mg /mL of the γ-PGA. However, this cell viability was

significantly increased by pretreatment with γ-PGA in a dose-dependent manner at the concentration range of 0.25-1 mg/mL. The cell viability reached up to 98.9% at 1mg/mL of the γ-PGA (Fig. 5B). In this study, our findings showed cytoprotective effect of γ-PGA against oxidative stress induced by H_2O_2 in Caco-2 cell without any cytotoxic effects at the tested concentration up to a sufficiently high concentration.

4. Conclusion

The ~400 kDa sodium salt γ-PGA purified from *Bacillus* sp. SJ-10 showed significant hydroxyl radical averting capacity by chelating transition metal ions and scavenging hydrogen peroxide (Fig. 8). Its averting capacity was maintained above about 90% during in vitro simulated gastrointestinal digestion model. The obtained result at least indicates that γ-PGA can stably protect the oxidative cell damage in gastrointestinal tract without inhibition of cell viability and cytotoxicity at a sufficiently high concentration of γ-PGA. In view of this, γ-PGA is a potential alternative for new dietary supplement and functional food. However, further research is needed in order to know more information about role of γ-PGA against oxidative damage in biological macromolecule, so actually in vivo analyses are being carried out.

Table and Figure

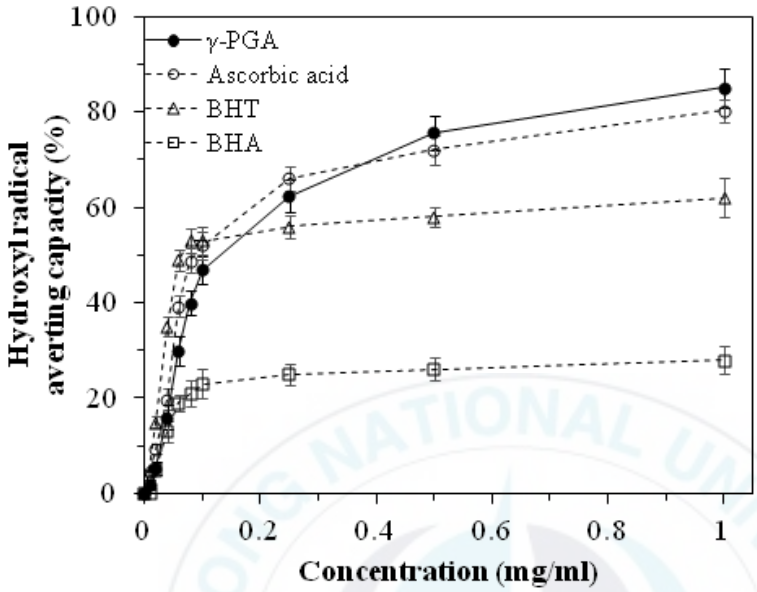

Fig. 1. Hydroxyl radical averting capacity of the γ-PGA with ascorbic acid, BHT and BHA as references.

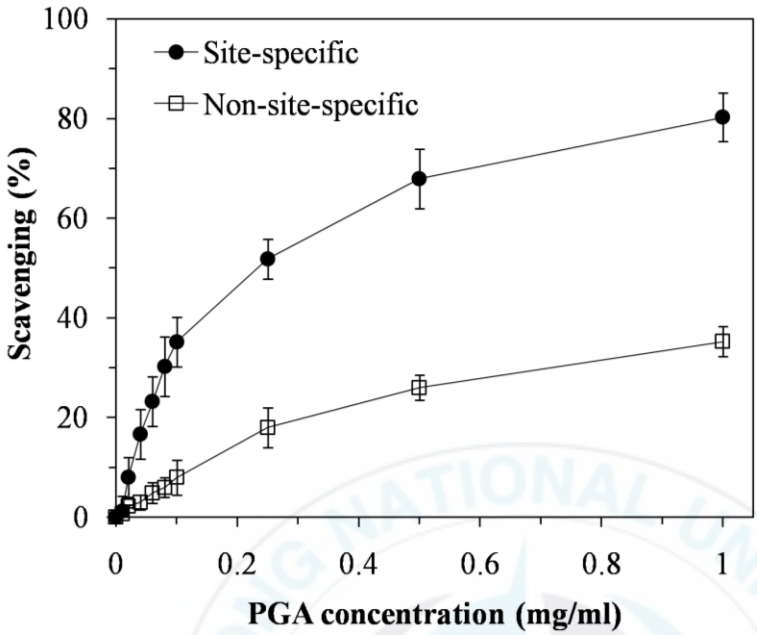

Fig. 2. Hydroxyl radical averting capacity of γ-PGA in site-specific and non-site-specific deoxyribose degradation assay.

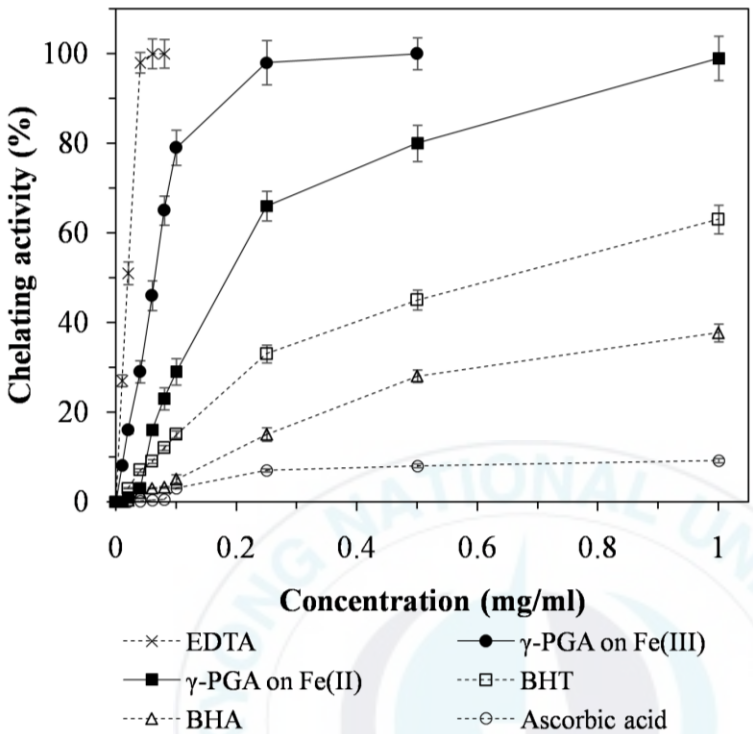

Fig. 3. Iron ions chelating effect of γ-PGA with EDTA, ascorbic acid, BHT and BHA as references.

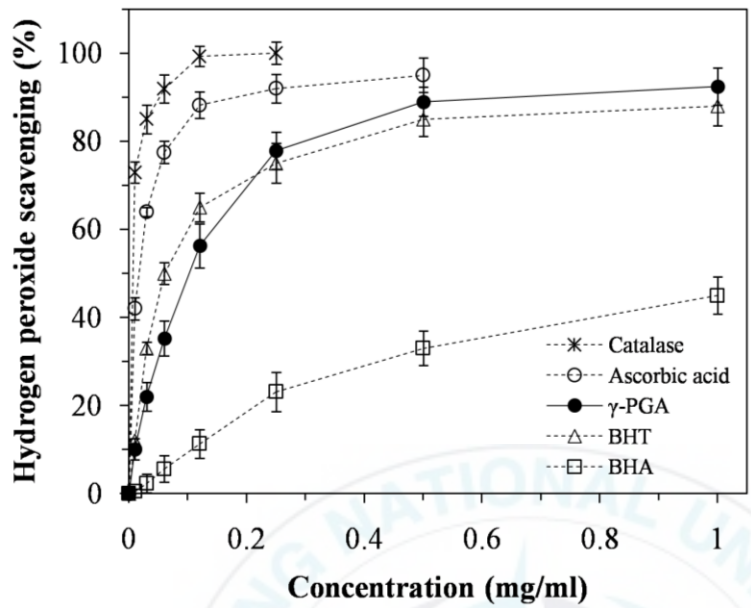

Fig. 4. Hydrogen peroxide scavenging activity of γ-PGA with catalase as a reference.

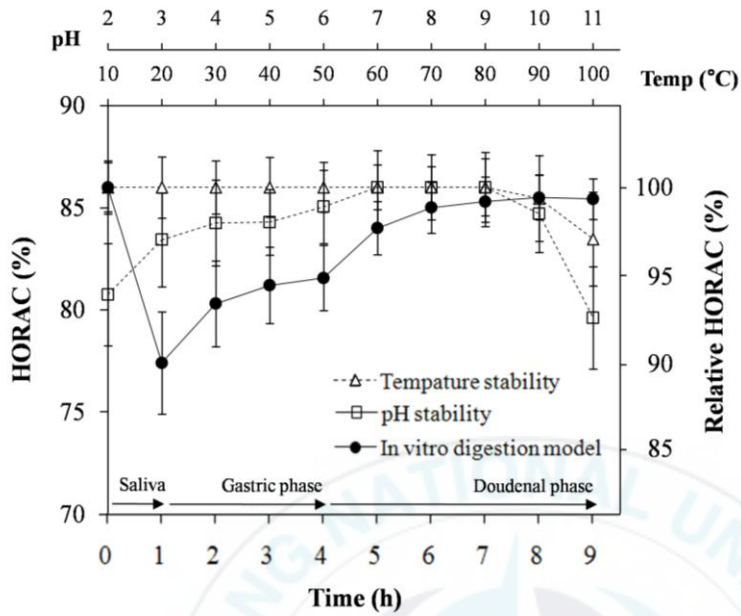

Fig. 5. pH and temperature stability and hydroxyl radical averting capacity of the γ-PGA *in vitro* digestion model.

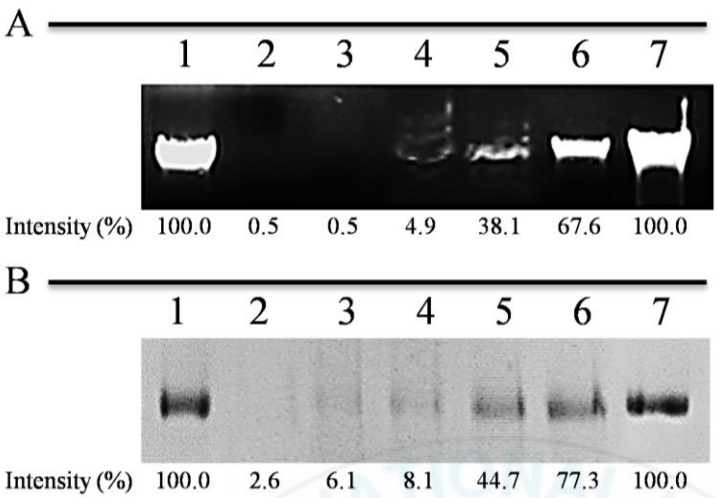

Fig. 6. Protective effect of γ-PGA on DNA (A) and protein (B) by •OH generated by Fenton reaction. (A) Lane 1, undamaged λ DNA (10μg); lane 2-7, λ DNA (10μg) + •OH + γ-PGA. The concentration of γ-PGA used are the following (in mg/ml): 0 (lane 2), 0.01 (lane 3), 0.1 (lane 4), 0.25 (lane 5), 0.5 (lane 6) and 1 mg/ml (lane 7). (B) Lane 1, BSA (50μg); lane 2-7, BSA (50μg) + •OH + γ-PGA. The concentration of γ-PGA used same concentration with (A).

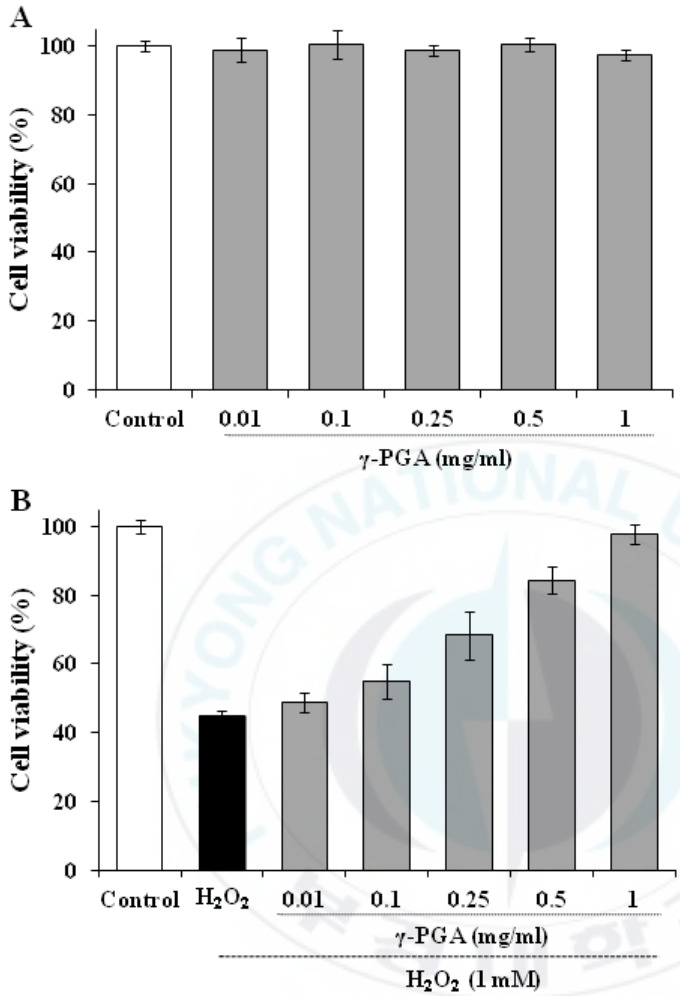

Fig. 7. Cytotoxicity (A) and cytoprotection (B) effects of γ-PGA on caco-2 cell.

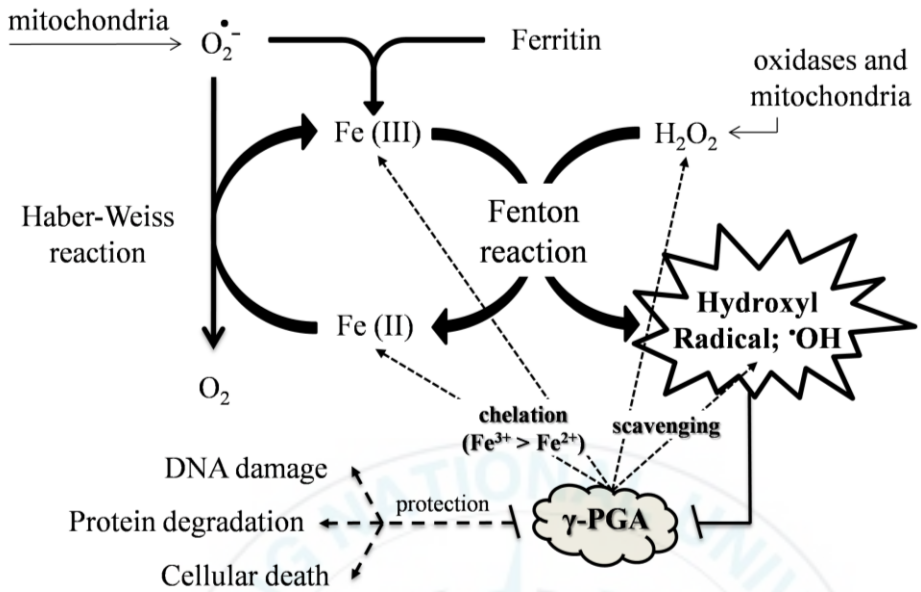

Fig. 8. Scheme of γ-PGA protective effect against hydroxyl radical induced damage where in the Haber-Weiss and Fenton reactions combined using poorly liganded iron in a catalytic cycle to produce the very damaging hydroxyl radical. This figure was slightly modified base on the previously reported by Prousek, J., 2007 [35].

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Summary (in Korean)

해양 유래 내염성 *Bacillus*가 생산하는 γ-PGA의 생화학적 효과 및 기능성에 관한 연구

이종민

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요약

본 연구는 우리나라 전통식품인 오징어 젓갈로부터 분리한 내염성 바실러스의 프로바이오틱스 특성을 평가하고, 균이 생산해내는 폴리감마글루탐산의 항산화 메카니즘과 하이드록시 라디칼에 의해 유발되는 산화적 손상으로부터 위장관 세포보호 효과에 대한 연구이다.

점질성 바이오폴리머인 폴리감마글루탐산을 생산해내는 본 균주의 프로바이오틱스 특성평가로서, 인간 장세포인 caco-2 세포에 대한 세포독성 및 용혈반응의 부재와 뛰어난 항생제 감수성으로 생균제로서의 안정성을 확인하였으며, 인체위장모델시스템을 통한 생존율 시험에서 산과 담즙산염에 대해 97% 이상의 생존률과, 세포 표면의 높은 소수성 및 강력한 응집능력으로 초기 접종 농도의 약 70%의 장부착능력을 보여주었다. 기능적인 측면에서, 그람 양성 및 음성 병원균에 광역 항균 스펙트럼을 가지고

있었으며, 특히 해양 유래 병원균인 *Edwardsiella*와 *Vibrio* 균주에 매우 강한 항균활성이 나타남을 확인하였다. 또한 강력한 항산화 활성과 β-galactosidase, phytase, bile salt hydrolase, catalase등의 기능성 효소의 생산과 더불어, 이눌린, 프럭도올리고당 뿐만 아니라 보리의 베타글루칸을 영양원으로 선택적으로 증식할 수 있는 프리바이오틱 가용능력으로, 본 균주는 동물과 인간의 프리바이오틱스로서 뿐만 아니라 수산 양식분야의 항생제 대체 생균제로서의 잠재력이 높음을 시사하였다.

본 균주가 생산해내는 세포외 점질성 바이오폴리머의 물리화학적 특성 조사를 위해 FT-IR, NMR, TGA 및 TLC를 이용하여 분석한 결과 대부분 L-glutamic acid 잔기로 이루어진 나트륨 염을 매개로 형성된 폴리감마글루탐산임을 확인하였다. 발효를 통해 고염의 조건에서 약 400kDa의 비교적 일정한 분자량의 폴리감마글루탐산을 생산하였고, 분자량과 관계없이 최고수율은 6% 염농도에서 약 26 g/L를, 400 kDa 분자량을 가지는 폴리감마글루탐산은 8% 염농도하에서 최대 24 g/L 수율을 보였다. 400kDa 분자량의 폴리감마글루탐산은 1 mg/mL 농도에서 85.2%의 하이드록시 라디칼 발생억제 능력을 가지고 있었으며 이는 발생되는 하이드록시 라디칼의 직접적인 소거능에 비해 전이금속의 킬레이션과 과산화수소 소거능을 통한 하이드록시 라디칼의 발생을 억제시키는 메카니즘으로 항산화 효과를 가짐을 본 연구를 통해 밝혔다. 하이드록시 라디칼 발생 억제 능력은 소화 위장관 모델에서 최종 약 95.3%의 활성을 유지 함으로서 구강 섭취시에도 큰 활성의 변화가 없음을 확인하였고, 1 mg/mL 농도에서 하이드록시 라디칼에 의해 발생되는 산화적 손상으로부터 DNA, protein 및 caco-2 cell을 완벽하게 보호하였다.

이러한 일련의 결과들을 통해 우리나라 전통 식품에서 분리한 안정성이 입증된 내

염성 바실러스가 생산하는 400 kDa의 폴리감마글루탐산은 식품, 사료 첨가제, 화장품 및 생물의학 분야에서 강력한 하이드록시 라디칼 억제제로서 항산화제와 세포보호제로서의 응용가능성을 보여주고 있으며, 본 연구는 일정한 분자량의 폴리감마글루탐산을 생산해내는 균주 및 생산방법에 관한 연구결과와 더불어 폴리감마글루탐산의 항산화 메커니즘을 밝힌 최초의 보고이다.

Acknowledgements

학위과정동안 아낌없는 격려와 세심한 배려로 올바르고 참다운 연구자의 길로 이끌어주신 공인수 교수님께 진심으로 머리 숙여 감사드립니다. 그리고 언제나 애정 어린 조언으로 관심 가져주신 홍용기 교수님, 김성구 교수님, 김중균 교수님, 이형호 교수님, 박남규 교수님, 정귀택 교수님께 감사 드립니다. 저의 부족한 학위 논문 지도와 연구자로서의 많은 가르침을 주신 국립수산과학원 김영옥 박사님, 동의대 이은우 교수님께 감사드립니다. 또한 부족한 후배를 지켜봐주시고 조언 아끼지 않으셨던 김동균 박사님, 실험실에서 동고동락하며 서로 좋은 연구 결과를 얻기 위해 고군분투 하였던 미생물유전공학 실험실 선후배님들에게도 감사한 마음을 전합니다. 지금의 제가 있기까지 너무 나도 많은 분들의 도움과 관심이 있었습니다. “이제 막 알에서 깨어 나온거야. 지금부터 시작이야.” 라는 공인수 교수님의 말씀 가슴깊이 새기어 항상 겸손한 자세로 매순간 최선을 다하며 가치있는 연구로 많은 이들에게 도움이 되는 연구자가 되겠습니다.

먼나라에서 힘들게 공부 하고 있을 내동생 수정이와, 한평생 아들, 딸의 앞길을 위해 당신들을 희생하시며 언제나 든든한 지원군이 되어주시는 아버지, 어머니께 죄송스런 마음과 감사한마음을 전합니다. 당신들이 있었기에 언제나 다시 일어 설수 있었습니다. 그리고 힘의 원천이 되어준 건아에게 고마움을 전합니다.